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**RATIONAL USE OF AZITHROMYCIN IN THE  
CONTROL OF TRACHOMA: USING QUANTITATIVE  
PCR TO ASSESS DISTRIBUTION OF INFECTION AND  
IMPACT OF TREATMENT**

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**Thesis submitted for the degree of Doctor of Philosophy in  
the University of London**

# ABSTRACT

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Trachoma is a chronic keratoconjunctivitis caused by ocular infection with *Chlamydia trachomatis* (CT). It is a major cause of blindness. This thesis addresses issues important for the rational use of the antibiotic azithromycin, one of the cornerstones of WHO's strategy for trachoma elimination.

An entire sub-village in Rombo District, Tanzania was invited to participate. Consenting individuals were examined and had swabs taken for quantitative PCR (directed against the single-copy CT gene *omp1*), at baseline (before treatment), and two, six, twelve and eighteen months after, mass distribution of single-dose azithromycin. At the time of drug distribution, both weight and height of each treated individual was recorded.

Four findings are highlighted. (1) Before treatment, and at each post-treatment time point, children below the age of ten years harboured the bulk of the community's ocular CT. Control programmes should view this age group as their principal target in antibiotic distribution campaigns.

(2) Signs of active trachoma were much less useful than age thresholds for predicting population subsets with heavy infections.

(3) Based on a summary statistic referred to as the community ocular CT load, or COCTL, the overall community burden of organism fell dramatically following azithromycin distribution, and stayed low for the entire eighteen months of follow-up. The COCTL was 13% of its baseline level at two months, 8% of baseline at six months, and 4% at both twelve and eighteen months. This may have been due to the high treatment coverage (98%) achieved.

(4) In the population studied, height was a good proxy for weight for determining azithromycin dose.

These studies contribute new information about the epidemiology of ocular CT infection, provide dramatic evidence of the potential effect of azithromycin when uptake is high, and should help to streamline azithromycin distribution efforts by simplifying determination of dose in the field.

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## INDEX OF SWAHILI WORDS

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<i>balozi</i>	(1) group of between eight and thirty-five (nominally ten) adjacent households; (2) the man or (much less commonly) woman who functions as the representative for the group
<i>kaya</i>	household
<i>kikope</i>	(1) a disease of the eye; (2) eyelid
<i>kitongoji</i>	sub-village
<i>mabalozi</i>	pl. of balozi
<i>mashamba</i>	pl. of shamba
<i>mganga</i>	traditional healer, medicine man
<i>mwalimu</i>	teacher
<i>shamba</i>	farm, garden, plot of cultivated ground
<i>vitongoji</i>	pl. of kitongoji
<i>wachawi</i>	sorcerers
<i>waganga</i>	pl. of mganga



## INDEX OF ABBREVIATIONS

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ACT	the ‘Azithromycin in Control of Trachoma’ trial (see Section 2.9.6, p.88)
AE	acridinium ester (see Section 2.6.6, p.52)
ATP	adenosine triphosphate
B/K	balozi / kaya (see index of swahili terms, and Section 5.2, p.104)
BMI	body mass index
CBM	Christoffel Blindenmission
CD	constant domain
CF	complement fixation
CI	confidence interval
CO	corneal opacity (see Section 2.5.4, p.40)
CMFL	community microfilarial load (see Section 5.9, p.124)
COCTL	community ocular <i>Chlamydia trachomatis</i> load (see Section 5.9.2, p. 125)
CPAF	chlamydial proteasome-like activity factor
CRP	cysteine-rich protein
CT	<i>Chlamydia trachomatis</i>
CTL	cytotoxic T lymphocyte
Da	daltons
d.f.	degrees of freedom
DFA	direct fluorescent antibody
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
EIA	enzyme immuno-assay
ELISA	enzyme-linked immuno-sorbent assay
FPC	follicles, papillae, cicatrices (see Section 2.5.4, p.40)
G+C	guanine and cytosine
GDP	gross domestic product
GET2020	global elimination of trachoma by the year 2020
GIS	geographic information system
GPS	global positioning system
HIV	human immunodeficiency virus
HSA	human serum albumin
<i>hsa</i>	gene coding for human serum albumin
hsp	heat shock protein
IC	internal control
ID	identity
IFN	interferon
IL	interleukin
ITI	International Trachoma Initiative
KCMC	Kilimanjaro Christian Medical Centre
LC	LightCycler
LCR	ligase chain reaction

LGV	lymphogranuloma venereum
LPS	lipopolysaccharide
LSHTM	London School of Hygiene & Tropical Medicine
MgCl <sub>2</sub>	magnesium chloride
MHC	major histocompatibility complex
MIF	microimmunofluorescence
mM	millimolar
MMX	master mix
MOMP	major outer membrane protein
MoPn	mouse pneumonia
NAAT	nucleic acid amplification test
NG	<i>Neisseria gonorrhoeae</i>
no.	number
OD	optical density
OMC	outer membrane complex
<i>omp1</i>	outer membrane protein 1: the <i>Chlamydia trachomatis</i> gene coding for MOMP
p.	page
PBP	penicillin binding protein
PCR	polymerase chain reaction
pCT	the common cryptic plasmid of <i>Chlamydia trachomatis</i>
PDGF	platelet-derived growth factor
Pmp	polymorphic membrane protein
POS	paediatric oral suspension
PPP US\$	purchasing power parity United States dollars
Q-PCR	quantitative PCR
RNA	ribonucleic acid
SAFE	Surgery, Antibiotics, Face washing, Environmental improvement (see Section 2.8, p.79)
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sp., spp.	species (singular), species (plural)
TALC	Teaching Aids at Low Cost
Taq	<i>Thermus aquaticus</i>
TF	trachomatous inflammation – follicular (see Section 2.5.4, p.40)
TI	trachomatous inflammation – intense (see Section 2.5.4, p.40)
T <sub>m</sub>	melting temperature
TS	trachomatous conjunctival scarring (see Section 2.5.4, p.40)
TT	trachomatis trichiasis (see Section 2.5.4, p.40)
UK	United Kingdom
UNICEF	United Nations Children’s Fund
USA	United States of America
VD	variable domain
WHO	World Health Organization

# 1. INTRODUCTION

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Infectious diseases confront man today as at no time since before the antibiotic era. Current estimates suggest that malaria kills approximately one million people each year in tropical Africa alone [1, 2]. HIV/AIDS, unrecognised before 1981, is now a global catastrophe [3, 4]. Tuberculosis is resurgent: one third of the world's population is probably infected with tubercle bacilli, which may be the world's leading killer of adults [5, 6]. Worldwide, annual child mortality from each of these three pathogens is overshadowed by that due to diarrhoeal disease, which is in turn eclipsed by the number of children who die yearly from acute lower respiratory tract infections [7, 8]. The emergence of new multi-drug resistant strains of *Mycobacterium tuberculosis* [9] and vancomycin- and epidemic methicillin-resistant *Staphylococcus aureus* [10-14]; the possible implications of global warming on the geographical range of tropical pathogens [6, 15]; the very recent threat of smallpox, ebola, anthrax, plague or tularaemia being used as instruments of bioterrorism [16-18]; and the sudden appearance of severe acute respiratory syndrome (SARS) [19, 20] have alarmed the medical community, governments, and the public [21-23] alike.

Amidst this cast of new and familiar foes competing for the attention of policy makers, research scientists and donors, one of the oldest – trachoma – suddenly, and perhaps transiently, looks vulnerable to attack. Pfizer's donation of an effective, single dose, well-tolerated, oral antibiotic for trachoma to six endemic countries has invigorated an international alliance against the disease – so much so that elimination of trachoma by the year 2020 is now a World Health Organization-supported target [24]. This provides a rare opportunity for optimism amongst those who take a public health approach to infectious disease control. It is critical that the momentum provided by Pfizer's donation is used to best effect. This is a good reason to choose trachoma as a priority subject for research, despite good claims from the other infections listed above. There is another reason, however, that is just as important.

Trachoma is a blinding disease, responsible for about 15% of global blindness [25]. It is, first and foremost, a disease of poverty: the millions of eyes blind from trachoma are set almost exclusively in the faces of the desperately poor – those least able to help themselves. The economic impact of blindness is huge in any environment, but for

someone whose family is already socially and economically vulnerable, it is devastating. Even if the suffering of the nearly 150 million people with clinically active trachoma is ignored, the effect of this disease on communities in which it is endemic is grave. Preventing trachoma blindness not only helps individual sufferers, but delivers enduring benefit to their families, to their village, and to future generations [26]. Trachoma has long been a neglected disease. We need effective interventions to eliminate it. The work presented in this thesis will hopefully contribute a little to their development.

## 2. REVIEW OF PREVIOUS WORK

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### 2.1 AETIOLOGY

Trachoma has been recognised for millennia as a blinding disease. It has been known in China for more than 4600 years, in the Middle East for 4000 years, and in Egypt for 3800 years [27-29]. Its contagious nature was recognised in Syria in the thirteenth century [30], but upon first coming to the attention of European surgeons during the Napoleonic campaigns in Egypt in 1798–99, the French determined that it was due either to sand or noxious night vapours. The British, on the other hand, believed that it was caused by a virus, and took appropriate measures; their infantry suffered a lower incidence of blindness [28]. In the late nineteenth and early twentieth century, the discovery of clinical trachoma in would-be US immigrants disembarking at Ellis Island, New York, was responsible for more than half of all medical detentions there, and meant certain deportation back to the port of origin [31, 32].

The causative agent of trachoma was not visualised until 1907, when Halberstaedter and von Prowazek described the presence of inclusion bodies (Halberstaedter-Prowazek bodies) inside infected cells. They believed the organism to be a protozoon [33]. The transmissibility of trachoma was by then already firmly established in the minds of the public. Hundreds of Russian and Austria-Hungarian First World War conscripts, for example, evaded military service by infecting their own eyes with discharges wiped from the eyes of trachoma patients [28]. Meanwhile, unconvinced by the findings of Halberstaedter and von Prowazek, researchers nominated a variety of bacteria, fungi, and viruses as the underlying pathogen [28]. It was not until 1957 in Peking that T'ang *et al.* completed the first successful isolation<sup>i</sup>, using chick embryos whose yolk sacs had been inoculated with material from infected human eyes [36]. They were able to serially passage the organism in eggs, and to use this material to infect the eyes of rhesus monkeys, producing characteristic clinical signs of trachoma and – on one occasion – inclusion bodies. Based on filtration experiments, they believed the trachoma agent to be a virus [36]. T'ang *et al.*'s methods were successfully replicated by Collier and Sowa in The Gambia in 1958 [37]. The isolates obtained were noted both to share a common antigen with, and to physically resemble, the agents of

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<sup>i</sup> Macchiavello claimed to have isolated the causative agent of trachoma in Ecuador in 1944 [34], by inoculating material from a 17-year old “who had suffered from a disease diagnosed as trachoma for three years” into the yolk sac of chick eggs. Because doubt was cast over the clinical diagnosis [35], however, his claim to first success has not been supported.

psittacosis<sup>ii</sup> and lymphogranuloma venereum [37], and the agent of some cases of cervicitis and mucopurulent conjunctivitis of the newborn [39].

As knowledge of the nature of these organisms accumulated, there was considerable debate over whether they represented a transitional remnant on the degenerate evolutionary pathway that Green had hypothesized [40] for the descent of viruses from bacteria, or whether they should be placed wholly within one or other of these classes. In 1966, Moulder published a comprehensive review of the growth, division, structure, chemical composition and metabolism of the group, taking into account the definitions of viruses and bacteria that had been recently proposed by Lwoff and Stanier respectively. He concluded fairly unequivocally that chlamydiae were intracellular bacteria, with a distinctive developmental cycle and unique structure [41]. The Taxonomy Committee of the American Society for Microbiology unified the organisms in the genus *Chlamydia*, and supported their status as bacteria [42]<sup>iii</sup>. Today, some fifteen major bacterial groupings are recognised, and the chlamydiae are the only ones whose members are all exclusively intracellular parasites of eukaryotes [43].

### 2.1.1 Developmental cycle

Chlamydiae lack cytochromes, so can not synthesise their own adenosine triphosphate (ATP). They are therefore obligate intracellular organisms, requiring energy-rich metabolic intermediates from host cells in order to complete their replication cycle [44]. To permit egress from infected cells and entry of new ones, a metabolically inert, extracellular, infectious form known as the elementary body (EB) alternates with the metabolically active, dividing, intracellular form (the reticulate body, or RB).

EBs of chlamydiae are spherical (or, rarely, pear-shaped) and 0.2–0.3  $\mu\text{m}$  in diameter [45]. They appear to bind to susceptible host cells via heparin bridges. There is considerable interest in identifying the chlamydial ligands involved in heparin binding: candidates include the major outer membrane protein, and the cysteine-rich protein OmcB, both of which are found in the chlamydial outer membrane complex (COMC) [46, 47]. More about both of these molecules (and about other COMC proteins) will follow in subsequent sections. Successful attachment of the EB is

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<sup>ii</sup> the isolation of which had been reported by Bedson and Western in 1930 [38]

<sup>iii</sup> The name *Chlamydia* Jones, Rake and Stearns, 1945 is derived from the Latin *chlamys*, which in turn is from the Greek *khlamus*: a short mantle fastened at the shoulder, worn by men in ancient Greece. It took priority over the

followed by its entry into the host cell. Though hosts are non-professional phagocytes, their oxidative and glycolytic pathways must be intact for entry to occur, suggesting that they participate actively in EB ingestion [48]. Tyrosine phosphorylation of host cell proteins and actin cytoskeletal rearrangement may be involved [49, 50]. Active participation of the EB, on the other hand, appears minimal: EB envelopes are internalised just as efficiently as whole EBs [51]. Processes involved in attachment and uptake may differ between the various species of chlamydiae, and even between variants of the same species [50, 52].

Once inside a host cell, the EB reorganises into a 0.5–1.0 µm diameter RB within a membrane-bound vacuole known as an inclusion. The RB grows and replicates by binary fission, remaining within the inclusion membrane (derived from the cytoplasmic membrane of the host) for the entire duration of the organism's intracellular phase. After a period of logarithmic multiplication, progeny differentiate back into EBs. A number of 'late phase' proteins are synthesised during the RB to EB transformation, including COMC proteins OmcB and OmcA, and two histone H1-like proteins, Hc1 and Hc2, which are involved in compaction of the chlamydial chromosome [45, 53]. EBs are released into the extracellular environment by fusion of the membrane of the inclusion with that of the host cell, or upon host cell lysis [44, 48]. The specific processes involved in cellular exit are poorly defined. In tissue culture, the entire developmental cycle from attachment to exit takes between forty-eight and seventy-two hours.

## **2.1.2 Structure and chemical composition**

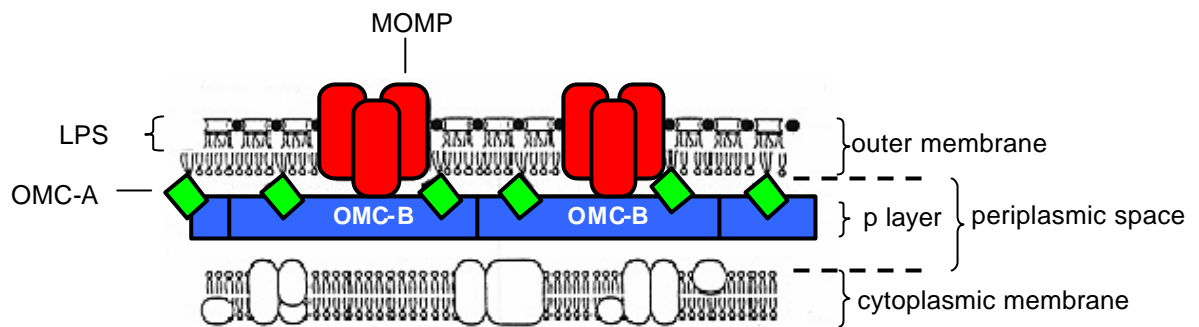
### **(a) Electron microscopy**

Using an electron microscope, an EB is seen to have a granular cytoplasm, reflecting the presence of 70S ribosomes, and an eccentrically-placed nucleoid containing condensed DNA [54]. The cell envelope is double-layered, resembling the cell envelope of gram-negative bacteria [44]. The cell wall (the portion of the cell envelope lying external to the cytoplasmic membrane) can itself be resolved into two layers: an inner ('p') layer composed of hexagonally arrayed structures with a periodicity of 16.7nm, and a granular outer layer containing the outer membrane [45, 54]. The inner layer therefore lies within the periplasmic space (Figure 2.1, p.23).

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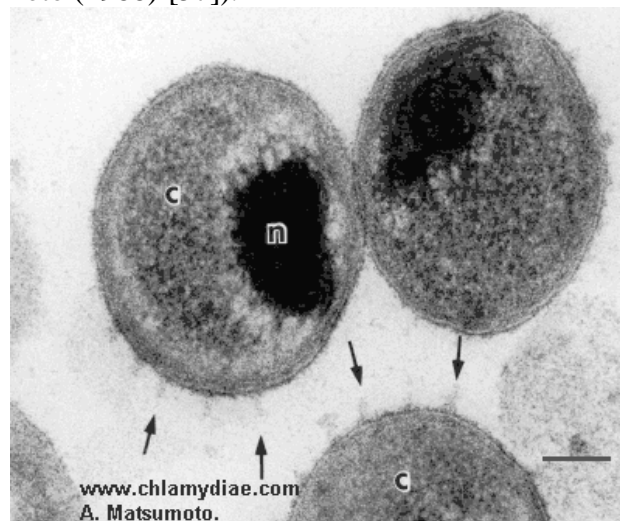
validly published names *Bedsonia* Meyer, 1953, and *Rakeia* Levaditi, Roger and Destombes, 1964, as well as over *Miyagawanella* Brumpt, 1938, which had not been validly published [42].

Figure 2.1: Model of the EB cell wall, after Everett and Hatch [55].



Cylindrical projections radiate from the outer membrane of the EB (Figure 2.2). Each projection is about 30nm long, and has its inner end at the cytoplasmic (inner) membrane, extending outwards to penetrate the outer membrane through the centre of a membrane-bound rosette. A rosette is made up of eight or nine protein subunits: the number of subunits varies between different species of chlamydiae. DNA strands can be seen connecting the nucleoid with the cytoplasmic membrane subjacent to the projections [54, 56].

Figure 2.2: Transmission electron micrograph of a chlamydial EB, showing projections (arrows). n=nucleoid; c=cytoplasm. The granulations in the cytoplasm are 70S ribosomes. The bar at the bottom right of the picture represents 0.15 $\mu$ m (Electron micrograph by Matsumoto (1988) [57]).



RBs are larger than EBs, and contain diffuse, fibrillar DNA plus a high concentration of ribosomes [58]. The cell envelope appears less complex than that of



the EB, lacking the hexagonally packed structures of the EB periplasmic space. The surface of the outer membrane, however, contains projections and rosettes at even higher density than are seen on EBs [58]. The outer end of the projections appear to contact the inclusion membrane, leading to the hypothesis that projection / rosette complexes have a secretory function analogous to the type III secretory system found in other bacterial species [45, 59].

#### **(b) Lack of peptidoglycan**

Most bacteria have peptidoglycan, a complex cross-linked polymer, in their cell envelope. In gram-negatives, peptidoglycan is found in the periplasmic space, while in gram-positives it lies immediately outside the cytoplasmic membrane and may comprise up to 50% of total cell wall material [44]. Its function is to help maintain cell shape and integrity despite the relatively high internal osmotic pressure of the bacterium. Penicillin and other  $\beta$ -lactam antibiotics inhibit growth of susceptible organisms by preventing formation of peptide cross-links in peptidoglycan. This effect is mediated through bacterial penicillin binding proteins (PBPs). Chlamydiae produce PBPs, and their growth is inhibited by penicillin, but – surprisingly – they do not appear to contain appreciable amounts of peptidoglycan [60, 61]. This paradox raises interesting questions about the biology of the organism [62, 63], and demands an alternative explanation for the rigidity and osmotic stability of the EB. These properties are currently thought to be conferred by the COMC.

#### **(c) Components, organisation and properties of the COMC**

The COMC was first defined in 1981, when Caldwell *et al.* [60] reported the outcome of their experiments with the detergent Sarkosyl<sup>iv</sup>, which had been shown to selectively solubilise the cytoplasm and cytoplasmic membranes of gram-negative bacteria [64]. Transmission electron microscopy of Sarkosyl-treated EBs showed empty EB particles with an apparently intact outer membrane [60]. Caldwell *et al.*'s method has since been used as the standard method for purification of the COMC. Using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of EB lysates, the same group identified a protein with a molecular weight of about 40kDa that was found in a number of chlamydial strains. They went on to show that this protein, which soon became known as the major outer membrane protein (MOMP), was one of the elements of the Sarkosyl-insoluble COMC, and that it comprised some 60% of the total protein mass of the EB cell wall. Two other research groups (Hatch *et al.* [65]; and

Salari and Ward [66]) independently discovered MOMP at around the same time [67]. <sup>125</sup>I-labelling of the protein indicated that it was surface exposed [60, 65, 66].

Hatch *et al.*, trying to extract MOMP from other components of the cell wall, noted that it could not be dissolved in SDS alone, or in mercaptoethanol alone, but was soluble in a solution containing both of these agents. This implied that disulfide bonds were important in binding MOMP into the COMC, and perhaps in maintaining the overall structural stability of the cell wall [65].

It was quickly recognised that the importance of MOMP was not confined to its structural role. Salari and Ward were able to extract MOMP from 14 of the 15 then-known serovars (see Section 2.1.4, p.27) of the chlamydial species *Chlamydia trachomatis* (see Section 2.1.3, p.26), and noted minor serovar-specific variation in its molecular weight [66]. The existence of species- and sub-species-specific epitopes within the protein was noted [68, 69], before it became clear that MOMP also contained serovar-specific epitopes [70, 71]. Even more importantly, polyclonal [72] and then monoclonal [73] antibodies to MOMP were shown to neutralise infectivity of the live organism. These discoveries raised hopes that the protein would be useful in the development of a protective subunit vaccine. Unfortunately, such hopes have so far remained unfulfilled [74].

Though multiple strands of evidence suggest that MOMP is surface exposed, other data have localised parts of the molecule to the periplasmic space [75]. These two conclusions are consistent with MOMP being an integral membrane protein. It now seems likely that MOMP's physiological function is as a membrane channel or 'porin' permeable to ATP. Prevention of uptake of host cell ATP could potentially be a mechanism by which antibodies to MOMP block cellular infection [76]. Although the protein is thought to form trimeric aggregates within the outer membrane [77], its actual conformation within intact chlamydiae is unknown [78].

Two other components of COMC have already been briefly mentioned: the cysteine rich proteins (CRPs) OmcB (57–62 kDa; also variously designated omp2, EnvB, large CRP, and CT443 [55, 79]) and OmcA (12–15 kDa; also – confusingly – designated omp3, EnvA, small CRP, and CT444 [55, 79]). The question of whether part of OmcB is surface localised is a matter of some debate. Evidence has been found that the protein can bind to tissue culture cells known to be susceptible to chlamydial infection [80], that it can be cleaved by application of trypsin to intact EBs [80, 81], that it binds heparin,

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<sup>iv</sup> sodium *N*-lauroyl sarcosine

and that monoclonal antibodies specific for the OmcB amino-terminal domain bind at the EB surface [47]. These observations are consistent with surface exposure, and a possible role in host cell attachment, as suggested earlier. Other studies, however, have found OmcB unaffected by trypsin [55], or failed to find surface-exposed epitopes by immunoelectron microscopy [82]. There seems to be consensus that the bulk of, if not all, the molecule is localised on the inner surface of the outer membrane [81], and it has been proposed that subunits composed of the carboxy-terminal portion of OmcB may make up the hexagonal array seen in the EB periplasmic space [45]. OmcA is a cysteine-rich lipoprotein that is not surface exposed, though it is probably attached to the outer membrane via its lipid moiety [45]. A model of the cell envelope structure has been suggested by Everett and Hatch [55] (Figure 2.1, p.23). It has been hypothesised that the structural stability of EBs is maintained by disulfide cross-linking between cysteine residues of MOMP, OmcB and OmcA [55, 83]. A similar supramolecular structure is absent from RBs, which are osmotically fragile. Protein cross-linking appears to occur during the last twenty-four hours of the intracellular phase of the life cycle [84].

Another family of proteins, the polymorphic membrane proteins (Pmps) was first identified by genetic analysis of a chlamydial strain that causes abortion in sheep [85]. Similar sequences were later described in other chlamydiae. Some of these proteins resemble autotransporters, but no evidence of secretory activity has yet been uncovered [45], and in the species causing human trachoma, only two of the nine Pmp proteins predicted on the basis of genome analysis have been identified as constituents of the COMC [86]. Alternative roles for particular Pmps have been proposed, including the suggestion that PmpD may be a cytolysin producing host cell rupture at the time of EB release [87]. The structure, intracellular location, regulation of expression, and likely function of this family of proteins is presently an area of energetic inquiry [45].

All species of chlamydiae identified to date share a common lipopolysaccharide (LPS) that differs from the LPS of other bacteria. The molecule is present in the outer membrane of the cell envelope throughout the life cycle [88-90], and contains polysaccharide epitopes recognised by the human humoral immune system [44, 91].

### **2.1.3 Taxonomy**

In 1980, when the Approved Lists of Bacterial Names were first published, the chlamydiae had two species: *Chlamydia trachomatis* (CT) and *Chlamydia psittaci* [92,

93]. In 1989, isolates previously identified as the TWAR strain of *C. psittaci* [94] were proposed as a third species, *Chlamydia pneumoniae*; it was differentiated from other chlamydiae on the basis of the shape of the elementary body, serology, and DNA analysis [95]. Another group of strains originally classified as *C. psittaci* were subsequently reassigned to *Chlamydia pecorum*, following further DNA and serological analyses [96].

In 1999, then, the bacterial family *Chlamydiales* contained one genus, *Chlamydia*, and four species: *C. pneumoniae*, *C. psittaci*, *C. trachomatis*, and *C. pecorum*. This classification enjoyed broad-based support. An emended classification based on ribosomal, biochemical, serological, ecological and DNA-DNA hybridization data was published by Everett *et al.* in the International Journal of Systematic Bacteriology in that year, reclassifying the family into two genera, *Chlamydia* and *Chlamydophila*, which together contain a total of nine species [43]. *C. trachomatis* strains naturally found in hosts other than man become new species within the genus *Chlamydia*. Subsequent phylogenetic analyses of five protein coding genes and the ribosomal operon are consistent with this revision [97, 98]. The new nomenclature has nevertheless proven controversial [99, 100]. Fortunately, whether or not the proposed changes enter general use, the designation of the pathogen responsible for human trachoma will remain *Chlamydia trachomatis*. Because the new nomenclature has not yet gained widespread acceptance, the old classification will be used as the basis for discussion here, with parenthetical reference made to the new classification where appropriate.

#### **2.1.4 Classification and tropism of *C. trachomatis* strains**

Three biovars (groups of strains distinguishable from others of the same species on the basis of physiological characteristics) of *C. trachomatis* were recognised before the Everett *et al.* revisions: mouse pneumonitis (MoPn), lymphogranuloma venereum (LGV), and trachoma.

The MoPn biovar gains species status (*C. muridarum* sp. nov.) in the new classification. Two strains have been identified: MoPn, which is found in the respiratory tract of mice, and SFPD, which has been isolated from the intestines of hamsters [43]. Neither strain is known to infect humans, and neither will feature prominently in this thesis.

The other two *C. trachomatis* biovars preferentially infect man. They are closely related, and have remained grouped as a single species in the reclassification of the chlamydiae [43]. There are four serotypes or serovars currently included in the LGV biovar, and fifteen in the trachoma biovar. Each of the nineteen can be distinguished from the others on the basis of binding affinity for monoclonal antibodies. They can also be differentiated by polymorphisms in the sequence of MOMP, or in the sequence of the gene *omp1*, which codes for MOMP (see below). There is very limited variation in these sequences between isolates of any given serovar [43]. Separation of LGV and trachoma strains into two biovars is based on tissue tropism: LGV strains can invade lymphatic tissue, while trachoma strains are restricted to mucosal epithelial cells.

The LGV serovars (L1, L2, L2a and L3) are rare. All are sexually transmitted, though the eye may also act as the portal of entry. Infection is associated with a suppurative adenitis, usually of the inguinal or perirectal nodes, as well as systemic symptoms. The disease is most commonly seen in tropical and subtropical areas [44].

The trachoma serovars of *C. trachomatis* are designated by the letters A through K, plus Ba, Da, Ia and Ja [43, 101]. Different serovars have different tissue preferences. Serovars A, B, Ba and C are the usual ocular isolates from patients with clinical trachoma in trachoma-endemic regions, while D to K, Da, Ia and Ja are typically associated with genital tract disease. The latter group are the commonest causes of urethritis and mucopurulent cervicitis in females, and non-gonococcal urethritis in males. They have also been linked to female pelvic inflammatory disease, infertility, ectopic pregnancy and chronic pelvic pain; male epididymitis, prostatitis and infertility; neonatal conjunctivitis and pneumonia; and various arthritides.

Strains are tissue selective, rather than specific. Even where trachoma is endemic, 'genital' serovars are found in the eye. Serovars D [102, 103, 104], E [103, 104], F [103-105], J [106], K [104] and L2 (with serovar A co-infection) [103], have all been isolated from conjunctival swabs taken from individuals with typical clinical signs of active trachoma. Similarly, 'ocular' *C. trachomatis* strains are not infrequently isolated from the genital tract. Frost *et al.* determined the serovars of 435 isolates taken from male and female attendees at sexually transmitted disease, perinatal and family planning clinics in Canada, and found that 5% were serovar Ba and 2% serovar C strains [107].

### 2.1.5 Genome of *C. trachomatis*

#### (a) Chromosome

*Chlamydia trachomatis* contains a single ~1,043,000 base-pair chromosome [108]. The first gene to be analysed was that coding for MOMP, which was designated *omp1*. In 1986, Stephens *et al.* sequenced *omp1* from a *C. trachomatis* L2 strain, after cloning and expressing the gene in an *Escherichia coli* ? bacteriophage [109]. Comparison of this gene with that from *C. trachomatis* serovars that were subsequently sequenced revealed extensive *omp1* sequence variation. Most of the polymorphisms were localised to four 40–90 base-pair-long variable domains (VDs), designated VD1, VD2, VD3 and VD4, regularly distributed amongst the relatively conserved constant domains (CDs). Examination of the accessibility of MOMP segments to digestion by proteolytic enzymes suggests surface exposure of VD-encoded peptide sequences, with localisation of the protein's amino- and carboxy-termini inside the periplasmic space [75]. Serovar specificity of CT appears to be determined by particular residues within VD1, VD2 and VD4 [75, 110]. Yuan *et al.* found that for each serovar, the VD coding for the most hydrophilic and charged amino acid sequence contained the serovar-specific epitope [111]. Later studies, though, indicate that *omp1* of a given serovar can incorporate multiple distinct serovar-specific epitopes, each of which may be found in a different VD [110]. Collectively, these findings (plus the demonstration that anti-MOMP antibodies neutralise the organism [72, 73], as discussed above) indicate that the *omp1* gene product, MOMP, spans the outer membrane of the cell envelope, and presents immunologically important epitopes, coded for by one or more VDs, at the cell surface. The likely biological function of this protein has already been discussed.

Heterogeneity in *omp1* constant domains between urogenital and trachoma isolates of the same Ba and C serovar has been identified. The altered nucleotide sequences produce changes in the amino acid sequences of MOMP, and could potentially play a role in determining tissue tropism or virulence of the organism [112]. More extensive analysis by Stothard *et al.* of sixty-nine strains representing seventeen serovars has not, however, supported an association between *omp1* sequence and tissue tropism, disease presentation, or epidemiologic success [101].

The first complete *C. trachomatis* genome sequence (a serovar D isolate) was published by Stephens *et al.* in 1998 [108, 113]. Notable findings included the localisation of an entire set of genes required for peptidoglycan synthesis (despite the lack of demonstrable peptidoglycan in the organism, as discussed above), genes

encoding ATP biosynthetic pathways (despite CT's apparent inability to make its own ATP), and nine genes encoding putative Pmps, paralogues of which had just been shown to be surface-exposed immunogens in a strain of *C. psittaci* (*Chlamydophila abortus* gen. nov., sp. nov.) pathogenic to sheep [114, 115]. The genome sequence of a *C. trachomatis* MoPn strain (*C. muridarum* sp. nov.) was subsequently also analysed, which highlighted the presence of a 'plasticity zone' near the chlamydial chromosome's origin of replication [116]. This zone includes genes coding for enzymes involved in tryptophan synthesis. Ocular but not genital serovars of the CT trachoma biovar have recently been found to carry a deletion or frame-shift mutation at this locus, rendering the former unable to synthesize tryptophan from exogenous indole [117, 118]. This finding is the first known point of difference in the biosynthetic capabilities of ocular and genital strains [117].

#### **(b) Plasmid**

In addition to the chromosome, chlamydiae commonly possess an extrachromosomal genetic element. The 7.4-kb plasmid pCT was first isolated from a *C. trachomatis* L2 strain by Palmer and Falkow in 1986 [119]. Their studies identified pCT DNA in laboratory strains of all the *C. trachomatis* serovars that cause human infection, as well as in 200 separate clinical isolates. The plasmid is very highly conserved, with less than 1% variation in nucleotide sequence [120, 121]. Because of this sequence conservation, and because maintenance of superfluous extrachromosomal DNA seems unlikely in a bacteria with a genome one quarter the size of that of *E. coli*, it was suggested that the plasmid might be essential for chlamydial growth or replication [119, 122]. However, several naturally-occurring *C. trachomatis* strains lacking the plasmid have since been isolated, including an L2 cultured from a patient with proctocolitis [123], a genotype B variant cultured from a male urethral swab [124], and a serovar E cultured from a male urethral swab [125]. Fortunately, such strains are thought to be rare [121], and no plasmid-free ocular isolates have been reported to date. Estimates for the mean number of plasmids per EB include 10 [119] (determined using a *C. trachomatis* L2 strain), 7–10 [126] (*C. trachomatis* L2), and 4 [127] (*C. trachomatis* L1). This estimate, and the possibility of chlamydial infection without the presence of plasmid DNA both have implications for determining the likely sensitivity of some laboratory assays for CT, as will be discussed later.

What, then, is the function of plasmid genes? They are expressed during chlamydial growth in eukaryotic cells [119]. Putative roles have been assigned to

protein products of several loci, but few definitive conclusions can be drawn [121]. Tantalisingly, antibodies to pgp3, one of the proteins encoded by pCT, have been found in a high proportion of sera from HIV positive individuals who lack other laboratory evidence of chlamydial infection [128], but are not found in healthy blood donors [129]. The significance of this finding is unclear.

## 2.2 NATURAL HISTORY AND CLINICAL FEATURES

Clinically, trachoma can be divided into its acute (active) and chronic or late-stage manifestations, but acute and chronic signs can occur at the same time in the same individual. In endemic areas, repeated episodes of active disease occur, particularly during childhood, and are probably required for later development of the chronic sequelae [130]. This concept will be briefly discussed in Section 2.4 (p.33).

The degree of distress caused by active ocular infection with *C. trachomatis* ranges from minimal to severe. Many infections are asymptomatic. In other cases, following an incubation period of 5–10 days, conjunctival infection produces an irritable, red eye, and scanty mucopurulent discharge. Involvement of the cornea in the acute inflammatory process can cause pain and photophobia [131]. In general, symptoms are milder than would be expected from the appearance of the eye [132].

The first *sign* of infection is a non-specific vasodilation of conjunctival blood vessels [131]. Specific changes may be noted after infection of two to four weeks duration, with the development of follicles subjacent to the conjunctivae of the fornices, the tarsal plates, and the limbus. Follicles are lymphoid germinal centres, and are found immediately below the epithelial cell layer. They are white, grey, translucent or yellow masses 0.2–3.0mm or more in diameter [133]; those with a diameter less than 0.5mm are not considered evidence of pathology. It is not uncommon to find one or two follicles in normal healthy eyes, usually towards the lateral or medial canthi. Because the superficial layer of the conjunctival stroma lacks lymphoid tissue until about three months after birth [134], newborns are unable to mount a follicular response to ocular chlamydial infection [135]. Papillae may also be noted at this stage: in mild cases, a few isolated, small, red dots can be seen with the naked eye. With the aid of a slit lamp, papillae appear as small swellings of the conjunctiva, each with a central vascular core. When inflammation is severe, an intense papillary reaction on the tarsal conjunctiva is associated with a diffuse thickening of the conjunctiva, obscuration of the deep tarsal vessels, and sometimes, eyelid oedema. If the cornea is involved in the inflammatory



process, a superficial punctate keratitis may be noted upon instillation of fluorescein into the conjunctival sac. Superficial infiltrates or pannus<sup>v</sup> also indicate corneal involvement. Follicles, papillae, and these corneal signs are features of active disease. The signs discussed below are all manifestations of late-stage trachoma.

Resolution of follicles is accompanied by scarring of the sub-epithelial conjunctiva. Scar deposition is most prominent in the upper tarsal plate, though the conjunctival fornices, the bulbar conjunctiva and the upper part of the cornea may also be involved. In trachoma-endemic areas, upper tarsal plate scar derived from repeated episodes of infection can eventually accumulate to such an extent that it becomes visible macroscopically after eversion of the upper lid, appearing as white bands against the erythematous background of the conjunctiva. At the limbus, replacement of follicles by scar results in the formation of depressions called Herbert's pits.

If sufficient scar accumulates, contraction of it over years will cause the upper eyelid to turn inward so that the lashes rub against the globe. This is known as trichiasis. When the whole lid margin is turned in, the condition is known as entropion. Scars around the bases of hair follicles can pull individual eyelashes into contact with the cornea, even without entropion [131]. Trichiasis is intensely irritating. Sufferers may use home-made forceps to remove their own lashes, or attempt to keep their lids elevated with strips of cloth tied round their heads.

Besides being painful, trichiasis damages the cornea. Collagenous scar is laid down as part of the repair process. Because scars are opaque, vision can be affected by scarring that involves the central part of the cornea.

## 2.3 PATHOLOGY

Histologically, active trachoma is characterised by the development of lymphoid follicles subjacent to the palpebral, forniceal and bulbar conjunctival epithelium. Follicles are dense collections of inflammatory cells, including B- and T-lymphocytes, plasma cells, monocytes, and macrophages. Mature follicles have pale-staining germinal centres surrounded by a mantle of proliferating lymphocytes. The nucleus of a germinal centre contains metabolically active plasma cells [133, 136, 137]. A diffuse infiltrate of plasma cells and polymorphonuclear lymphocytes, as well as some macrophages, mast cells, and eosinophils, is seen in the conjunctival stroma between

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<sup>v</sup> Subepithelial infiltration of fibrovascular tissue into the peripheral cornea. Duke-Elder believed that "some degree of pannus is present in every case of trachoma" [28](p.1609).

follicles, and between follicles and the basement membrane of the epithelium. Both CD8<sup>+</sup> and CD4<sup>+</sup> T-lymphocytes are recruited as part of the inflammatory response to acute infection, with CD8<sup>+</sup> T-cells predominating [138]. Stromal capillaries are dilated and engorged. A band of plasma cells lines the deep surface of the basement membrane and surrounds the acini of accessory lacrimal glands. A few dendritic cells can be found at the boundary between stroma and epithelium. The epithelium itself is generally hyperplastic, though thinning is observed over follicles. Infected cells contain inclusions [136, 137, 139].

In the conjunctivae of patients with late-stage trachoma (conjunctival scarring with or without trichiasis or entropion), inclusion bodies are rarely seen. The conjunctival epithelium is atrophic, with a reduction in the number of columnar epithelial cells; goblet cells are absent [139, 140]. Infoldings of epithelium may form retention cysts (pseudocysts of Henle's glands) containing inspissated secretions [140]. There is a proliferation of fibroblasts, and deposition of vertically oriented, parallel collagen fibres in a layer adherent to the tarsal plate. Newly-deposited collagen is predominantly of type V, which is absent from the normal conjunctiva [141]. This scar tissue replaces the normal thin, loose, vascular stroma, and causes apparent thickening of the tarsus [140]. Meibomian glands are infiltrated by lymphocytes, and are atrophic or deformed by scarring, with thickening of the basement membrane around the germinative cell layer [139, 140]. Plasma cells are rare in the stroma of patients with scarring trachoma, unless there is concurrent active trachoma [142, 143]. The T-lymphocytes found in the Meibomian gland epithelia and stroma of these cases are predominantly CD8<sup>+</sup>. It has been speculated that the CD8<sup>+</sup> lymphocytes are suppressor T-cells involved in down-regulating the anti-chlamydial immune response [143].

In the scarred cornea, there is degeneration and irregularity in the thickness of the epithelium. Bowman's membrane may be destroyed. There is disorganisation of the normal perpendicular orientation of the collagen lamellae in the stroma, sometimes with calcification. Capillaries or ghost vessels may be seen in the superficial and middle stromal layers [144, 145].

## **2.4 IMMUNITY AND PATHOGENESIS**

In some areas hyperendemic for trachoma, most children have signs of active disease [146, 147]. Only a small proportion go on to develop trachomatous corneal

opacity. A brief outline of current concepts in immunity to ocular chlamydial infection and the pathogenesis of trachoma blindness is presented below.

#### **2.4.1 Factors attributable to the host immune response**

The innate immune system responds to the presence of chlamydiae. There is an influx of neutrophils to the site of infection, and production of pro-inflammatory cytokines, including interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , platelet-derived growth factor (PDGF) and tumour necrosis factor (TNF)- $\alpha$ , by infected epithelial cells and conjunctival macrophages [148-150]. Normal conjunctival cells do not express IL-1 [150]. Infected cells, on the other hand, release cytokines continuously from about 20 hours post EB-entry; additionally, released IL-1 $\alpha$  seems able to stimulate cytokine production in adjacent, uninfected cells [151]. Based on animal models, it has been suggested that a more florid innate response in the early stages of infection is associated with a shorter duration, less intense infection, and therefore an improved outcome [149]. Unfortunately, inflammatory responses may be responsible for some of the tissue damage attributed to infection, and may not necessarily clear the organism from the epithelium. In a case-control study in The Gambia, subjects with scarring trachoma were significantly more likely than controls to have TNF- $\alpha$  detectable in tears, and to have a particular allele (-308A) of the TNF- $\alpha$  gene promoter [152].

Strain-specific protective immunity against ocular or genital *C. trachomatis* infection seems to be induced by repeated infection [153, 154]. In trachoma-endemic communities, induction of anti-chlamydial immunity seems likely to underlie the observed decrease in the duration of both infection and disease with age, and the increase in intensity of inflammation within the shorter disease episodes of older people [155]. Precise mechanisms for the development of the adaptive immune response are unclear; a brief description of conclusions drawn on current data is made difficult by the variety of *in vitro* and *in vivo* models used, and the fact that responses to primary infection and reinfection appear to differ [156]. Cytokines released as part of the innate response (which are predominantly of the TH-1 type) likely contribute to initiating adaptive immunity, and both cell-mediated and antibody-mediated systems seem to be recruited. Local mucosal and circulating antibodies are both produced [157]. MOMP-specific antibodies are the first to be detectable [157]; as noted earlier, anti-MOMP antibodies can block EB attachment and internalisation. Unfortunately, variant strains that have MOMP sequences differing by as little as one amino acid are uninhibited by

monoclonal or polyclonal antibodies that effectively neutralise the type strain [158]. It seems likely that antibodies make a modest contribution to, but are not essential for, protection [156]. It is also possible that anti-chlamydial IgG in tears actually enhances the infectivity of CT for human conjunctival epithelium [159].

Cell mediated immune mechanisms (the TH-1 response), and CD4+ cells in particular, seem to be critical in clearing infection [156, 160]. There is evidence that individuals who develop scarring do not have adequate anti-chlamydial TH-1 responses. In lymphocyte proliferation assays [161], peripheral blood mononuclear cells of individuals with conjunctival scarring have reduced responses to CT antigens compared to matched control subjects, but equivalent responses to other antigens [162].

CD4+ T-lymphocytes recognise antigens presented in association with class II major histocompatibility complex (MHC) molecules. Generally, class II molecules are not found on the conjunctival epithelium. In trachoma-endemic communities, however, conjunctival epithelial cells of patients with ocular CT infection express class II MHCs [163]. In animal models, there is a marked increase in the number of CD4+ cells in conjunctival tissue after experimental inoculation with CT [138].

The TH-1 cytokine interferon (IFN)- $\gamma$  has an uncertain role [149]. It has been demonstrated to inhibit the growth of CT *in vitro* and *in vivo*, is important in mice for the clearance of CT infection [164], and is probably responsible for inducing expression of MHC class II antigens on conjunctival epithelial cells in infected humans [163]. However, it may also induce a state of chlamydial latency within the epithelium, or have a direct cytotoxic action on infected cells [165]. CD8+ cytotoxic T lymphocytes (CTLs), which recognise antigen presented by class I MHC molecules (found on all nucleated cells and platelets) can lyse infected cells and produce IFN- $\gamma$  and other cytokines. CT-specific CTLs have been identified. Injection of this cell line into immuno-naïve mice can protect them against challenge with *C. trachomatis*. This effect is dependent on both CTL-derived and non-CTL-derived IFN- $\gamma$  [164, 166].

### **1.1.1 Factors attributable to the organism**

There is little to suggest that there are major differences in inherent virulence between different ocular serovars of CT, though possible strain variation in susceptibility to IFN- $\gamma$  has been hypothesised [167]. Several species-specific characteristics deserve comment.

Firstly, intracellular chlamydiae inhibit phagosome-lysosome fusion by mechanisms that are yet to be defined [51]. This allows the organism to survive and replicate within conjunctival epithelial cells.

Secondly, laboratory test-detectable infection sometimes persists in human conjunctival tissue after the resolution of (or without) clinical signs [168, 169]. This can be termed ‘persistent’ infection; unfortunately, there is no consistency in the literature in the use of this term. Additionally, interrupted chlamydial development with resultant chronic, laboratory test negative, ‘cryptic’ infection has been documented *in vitro* [170-172]: if such a phenomenon occurs *in vivo*, it is possible that a clinically quiet, test negative human conjunctiva could nevertheless have a chronic *C. trachomatis* infection. The natural history of infection and its relationship with clinical disease will be mentioned again in Section 2.6.12(iii) (p.66).

If a CT strain is able to establish chronic infection – regardless of the associated clinical and laboratory profile – it represents either failure or subversion of the adaptive immune response. It seems reasonable to assume that the greater the duration (and intensity) of conjunctival infection, the greater the exposure of conjunctival tissues to putative CT-derived pro-inflammatory factors. Three such candidate molecules will be briefly described.

(1) The chlamydial heat shock protein (hsp)-60 is homologous to HtpB of *Coxiella burnetii*, GroEL of *E. coli*, and Hsp60 of *Mycobacterium tuberculosis* [173]. It induces a delayed-type hypersensitivity response in the conjunctivae of previously sensitised experimental animals [174]. In humans, serum antibodies to this protein have been associated with scarring, though whether anti-hsp-60 itself contributes to immunopathology or is simply found more commonly in people with long standing infection is not known [175].

(2) In addition to genes for tryptophan synthesis, the plasticity zone of the CT chromosome contains genes encoding proteins homologous to the large cytotoxins A and B of *Clostridium difficile* [176]. Protein is preformed within EBs at the time of cellular infection, and induces host cell rounding and cytoskeletal collapse – changes identical to those induced by clostridial cytotoxin B. These putative CT cytotoxins also have homology to the *E. coli* lymphostatin protein LifA, which inhibits lymphocyte activation. Whether or not they are involved in mediating immune evasion in the human eye is presently unclear [176].

(3) Chlamydial proteasome-like activity factor, or CPAF, is a recently identified protein secreted into the host cell cytoplasm [177]. It may be involved in down-regulating expression of IFN- $\gamma$ -inducible MHC class II [178], and constitutive and IFN- $\gamma$ -inducible expression of MHC class I [179] molecules by infected cells. It may therefore help the organism to evade immune recognition.

### 1.1.2 Pressure of infection

A single episode of CT-stimulated inflammation resolves in weeks or a few months, even without treatment [155]. Conjunctival scarring produced by a single infection is unlikely to produce trichiasis. Grayston *et al.* [130] believed that repeated re-infection over a number of years was required for the development of cicatricial complications. In trachoma hyperendemic areas, however, recrudescence or reinfection occurs within weeks or months of resolution of the previous episode of active disease [180-182].

The number, duration and intensity of infections required for the accumulation of significant scar in the human conjunctiva is not known. Laboratory and natural experiments suggest that intermittent exposure, or even intense exposure of brief duration, is insufficient:

First, eleven inoculations of live strains of *C. trachomatis* into the eyes of a near-blind human volunteer over a seven year period, in addition to repeated challenge with various heat-inactivated chlamydial materials, was not associated with clinical sequelae, even after twenty years of follow-up [183]. Second, in 1965, Detels *et al.* [184] reported the prevalence of clinical signs of trachoma in communities of Punjabi Indians in British Columbia. The individuals examined, or their parents or grandparents, had all emigrated from Punjab State in the years between 1905 and 1964. Before and during that period, trachoma was hyperendemic in northern India [185]. Signs of trachoma were rarely found in members of the community who were Canadian-born, suggesting that the incidence of transmission in the displaced communities was low. In Indian-born subjects over thirty years of age, the prevalence of blinding sequelae was 8% amongst those who had lived in India for less than twenty years before emigrating, and 23% amongst those who had lived in India for between 20 and 39 year<sup>vi</sup>.

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<sup>vi</sup> Insufficient data are provided in the paper to permit adjustment for age, which is a likely confounder in this association.

In contrast, progression of disease and development of blinding sequelae is well documented in individuals who remain in hyperendemic areas. Nearly 30% of a cohort of ninety-eight Tanzanian pre-school children who had severe inflammatory trachoma (TI – see Section 2.5.4(ii), p.43) on at least three of four examinations in 1989 had developed visible scarring at follow-up seven years later [186]. Of 523 adult women with visible conjunctival scarring at baseline, forty-eight (9.2%) had developed trichiasis within seven years: an average incidence of 1.3% per year for that cohort [187].

Presumably, as Emerson *et al.* [188] have suggested, there is a threshold transmission intensity below which trachoma will cease to cause blindness in a community. Exactly where this threshold lies, or even how it could be measured, are presently matters only for speculation.

#### **2.4.4 Pathogenesis of corneal opacity**

Corneal damage by in-turned eyelashes is generally assumed to be the ultimate mechanism for blindness due to trachoma. Unsurprisingly, then, trichiasis is a risk factor for developing corneal scar. A longitudinal study of patients with cicatricial trachoma in The Gambia showed the presence of trichiasis at baseline to increase seven-fold the risk of corneal opacity twelve years later, compared to those who had scarring but not trichiasis at baseline [189].

It seems likely that anatomical factors, such as the position of the insertion of the pretarsal orbicularis (which will affect the direction of its pull on the lid), and the relative strength of the marginal fibres of this muscle in comparison to the pretarsal portion (which may determine the risk of tarsal plate inversion) will help to determine how quickly corneal scar accumulates in patients who have conjunctival scar or trichiasis [190]. Other features of the trachomatous eye likely also contribute. Scarring of forniceal-mucous, lacrimal and Meibomian glands reduces their secretory output and disturbs the tear-film architecture, drying the eye. Corneal drying accelerates epitheliopathy. The concentrations of lactoferrin and lysozyme in tear fluid are reduced [191], presumably due to a disproportionate reduction in the aqueous phase. All of these changes, particularly when added to repetitive epithelial injury from misdirected eyelashes, predispose the cornea to secondary bacterial and fungal infection. Such infections generate an added burden of corneal scar. The use of traditional eye medicines may also contribute to corneal damage [192].

## **2.5 CLINICAL DIAGNOSIS**

### **2.5.1 History**

Because active trachoma is usually found in children, is an almost universal experience of residents of hyperendemic communities, and seems to cause little discomfort, there are generally few reported symptoms. Individuals with trichiasis can be symptomatic. The degree of distress depends on the number of lashes touching the globe, whether or not the cornea is abraded, and whether there is associated blepharospasm. Symptoms have been described above in the paragraphs (Section 2.2, p.31) on natural history.

### **2.5.2 Examination**

Examination of the eye for the clinical signs of trachoma involves the careful inspection of the lashes, cornea and limbus, then eversion of the upper lid and inspection of the tarsal conjunctiva. Binocular magnifying loupes (2.5×) and adequate lighting are required. Signs of interest are as described in Section 2.2 (p.31).

### **2.5.3 Differential diagnosis**

Follicles are not pathognomonic for trachoma, but are a reasonably specific sign for it when seen in individuals living in trachoma-endemic communities. The differential diagnosis of follicular conjunctivitis includes adult inclusion conjunctivitis (caused by infection with urogenital strains of *C. trachomatis*); other bacterial infections, particularly *Moraxella spp.*, *Streptococcus pneumoniae* and *Borrelia burgdorferi*; viral infections, including adenovirus, molluscum contagiosum, and HSV; pediculosis palpebrarum; and toxic conjunctivitis secondary to topical drugs or eye cosmetics. The giant ‘cobblestone’ papillae of vernal keratoconjunctivitis (‘spring catarrh’) may be mistaken for follicles at first glance, but are both clinically and histologically distinct [134, 135, 193].

Papillae are poorly specific for trachoma. They form part of the conjunctival tissue’s response to many acute and chronic inflammatory disorders, and are therefore seen in bacterial, viral and allergic conjunctivides.

In trachoma-endemic areas, pannus, conjunctival scarring and trichiasis are nearly always attributable to trachoma. Herbert’s pits are pathognomonic of past trachomatous inflammation. Corneal opacity, though, may result from a variety of causes, including previous traumatic injury, severe measles, herpes simplex keratitis, or corneal ulceration



of any cause: considered alone, the prevalence of corneal scar will overestimate the contribution of trachoma to the total burden of blindness and visual impairment.

#### **2.5.4 Grading systems**

Grading systems are used in an effort to standardise diagnosis in field surveys and research studies. In literature in English since 1900, at least ten different complete systems or variations on systems have been published: by MacCallan, 1908 [194]; MacCallan, 1931 [27]; the WHO Expert Committee on Trachoma, 1962 [195]; the Fourth WHO Scientific Group on Trachoma Research, 1966 [196]; Tarizzo, 1973 [197]; Dawson, Jones and Darougar, 1975 [198]; Dawson, Jones and Tarizzo, 1981 [132]; Darougar and Jones [133]; Tielsch, West, Johnson, Tizazu, Schwab, Chirambo and Taylor, 1987 [199]; and Thylefors, Dawson, Jones, West and Taylor, 1987 [200]. (In addition, Sarkies, 1965 [201] contributed a three-stage classification of trachomatous entropion, which has been rarely, if ever, used by other authors.) The several variants of the MacCallan trachoma classification [27, 194] were probably derived from a description of the ‘four stages of trachoma’ by Aetius of Amida in the sixth century [202], and were durably popular in the ophthalmological literature of the first half of the twentieth century<sup>vii</sup>, despite (a) implying that the clinical course is linear, ignoring trachoma’s multicyclic nature; (b) not mentioning corneal opacity or visual impairment, and therefore having little prognostic value; and (c) lacking clear-cut definitions that would allow one ‘stage’ to be reliably differentiated from the next [29, 132, 133, 198]. Some of the later schemes are exceptionally complex: the 1966 proposal by the Fourth WHO Scientific Group on Trachoma Research, for example, took more than four pages to outline, recommending the grading of up to nineteen signs, each of which had its own scale [196]. Two classifications are in current general use and will be discussed here. The others have largely been superseded.

##### **(i) Dawson, Jones and Tarizzo, 1981 (‘the modified WHO system’ or ‘the FPC system’)**

This grading system was developed “to describe more precisely the intensity of active trachoma” [198](p.279) than did the MacCallan classification. An embryonic

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<sup>vii</sup> The MacCallan system still occasionally appears in papers in peer-reviewed journals: in 2000, the Paediatric Infectious Diseases Journal published the results of a Senegalese survey that used the MacCallan scheme [203]. It is also described (alongside the WHO simplified system – see main text) in the Wills Eye Manual 3<sup>rd</sup> edition (1999), a

version of the FPC system can be found in the 1962 WHO Expert Committee on Trachoma Third Report [195]. Its evolution can be traced through a number of subsequent publications [197, 198], before its appearance in final (for WHO<sup>viii</sup>) form in the 1981 WHO manual “Guide to trachoma control in programmes for the prevention of blindness” [132]. The modified system includes five signs, each of which is graded independently in the right and left eye, as follows:

#### Upper tarsal follicles (F)

- F<sub>0</sub> No follicles
- F<sub>1</sub> Follicles present, but no more than 5 in zones 2 and 3 together<sup>ix</sup>
- F<sub>2</sub> More than 5 follicles in zones 2 and 3 together, but less than 5 in zone 3
- F<sub>3</sub> Five or more follicles in each of the three zones

#### Upper tarsal papillary hypertrophy and diffuse infiltration (P)

- P<sub>0</sub> Absent: normal appearance
- P<sub>1</sub> Minimal: individual vascular tufts (papillae) prominent, but deep subconjunctival vessels on the tarsus not obscured
- P<sub>2</sub> Moderate: more prominent papillae, and normal vessels appear hazy, even when seen by the naked eye
- P<sub>3</sub> Pronounced: conjunctiva thickened and opaque, normal vessels on the tarsus are hidden over more than half of the surface

#### Conjunctival scarring (C)

- C<sub>0</sub> No scarring on the conjunctiva
- C<sub>1</sub> Mild: fine scattered scars on the upper tarsal conjunctiva, or scars on the other parts of the conjunctiva
- C<sub>2</sub> Moderate: more severe scarring but without shortening or distortion of the upper tarsus

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book intended as a consulting room reference for ophthalmologists, emergency room physicians, primary care physicians and medical students [204].

<sup>viii</sup> Darougar and Jones published a minimally altered version of the system in 1983 [133]. For interobserver trials conducted in 1987, Tielsch *et al.* [199] used a different version with (other) minor changes.

<sup>ix</sup> “For scoring follicles, the upper tarsal conjunctival surface is divided into three approximately equal zones. These zones are defined by two imaginary lines which, as viewed on the everted tarsal surface, are approximately parallel with the upper tarsal border and curve upward towards their lateral extremities... Zone 1 includes the entire upper tarsal border and adjacent lateral tarsal surface; zone 2 occupies the area between zones 1 and 3 and extends to the lateral quarters of the lid margin; zone 3 includes the tarsal conjunctiva adjacent to the central half of the lid margin and, at its centre, covers just less than half the vertical extent of the tarsal surface.” [132](p.14).

C<sub>3</sub> Severe: scarring with distortion of the upper tarsus

Trichiasis and/or entropion (T/E)

T/E<sub>0</sub> No trichiasis or entropion

T/E<sub>1</sub> Lashes deviated towards the eye but not touching the globe

T/E<sub>2</sub> Lashes touching the globe but not rubbing on the cornea

T/E<sub>3</sub> Lashes constantly rubbing on the cornea

Corneal scarring (CC)

CC<sub>0</sub> Absent

CC<sub>1</sub> Minimal scarring or opacity but not involving the visual axis, and with clear central cornea

CC<sub>2</sub> Moderate scarring or opacity involving the visual axis, with the pupillary margin visible through the opacity

CC<sub>3</sub> Severe central scarring or opacity with the pupillary margin not visible through the opacity

The system selects the upper tarsal conjunctiva to provide an “index of trachomatous inflammation in the eye as a whole” [132](p.14). The intensity of inflammation is classified as trivial, mild, moderate or severe using Table 2.1, which is reproduced here as it appears in the WHO guide. In that document, the meaning of “key sign” is not explained.

Table 2.1: "Intensity of inflammation" classification scheme proposed by Dawson *et al.* [132](p.14)

Intensity	Follicles	Papillae	Key sign
Severe	F <sub>3</sub> (or F <sub>2</sub> or F <sub>1</sub> ) <sup>x</sup>	P <sub>3</sub>	P <sub>3</sub>
Moderate	F <sub>3</sub>	P <sub>2</sub>	F <sub>3</sub>
Mild	F <sub>2</sub>	P <sub>0</sub> , P <sub>1</sub> or P <sub>2</sub>	F <sub>2</sub>
Trivial (insignificant or absent)	F <sub>0</sub> or F <sub>1</sub>	P <sub>0</sub> , P <sub>1</sub> or P <sub>2</sub>	F <sub>0</sub> or F <sub>1</sub>

Using this classification system (with a few minor alterations), Tielsch *et al.* [199] found the intra- and inter-observer agreement of four well-trained, experienced ophthalmologists working in the field to be variable and often poor. For non-specialist

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<sup>x</sup> “The follicles may be obscured by severe papillary hypertrophy and diffuse infiltration (P<sub>3</sub>)” [132] p.14.

health personnel, the modified WHO system is thought to be too complex [200]. However, it still enjoys a degree of popularity with some experts [205-207].

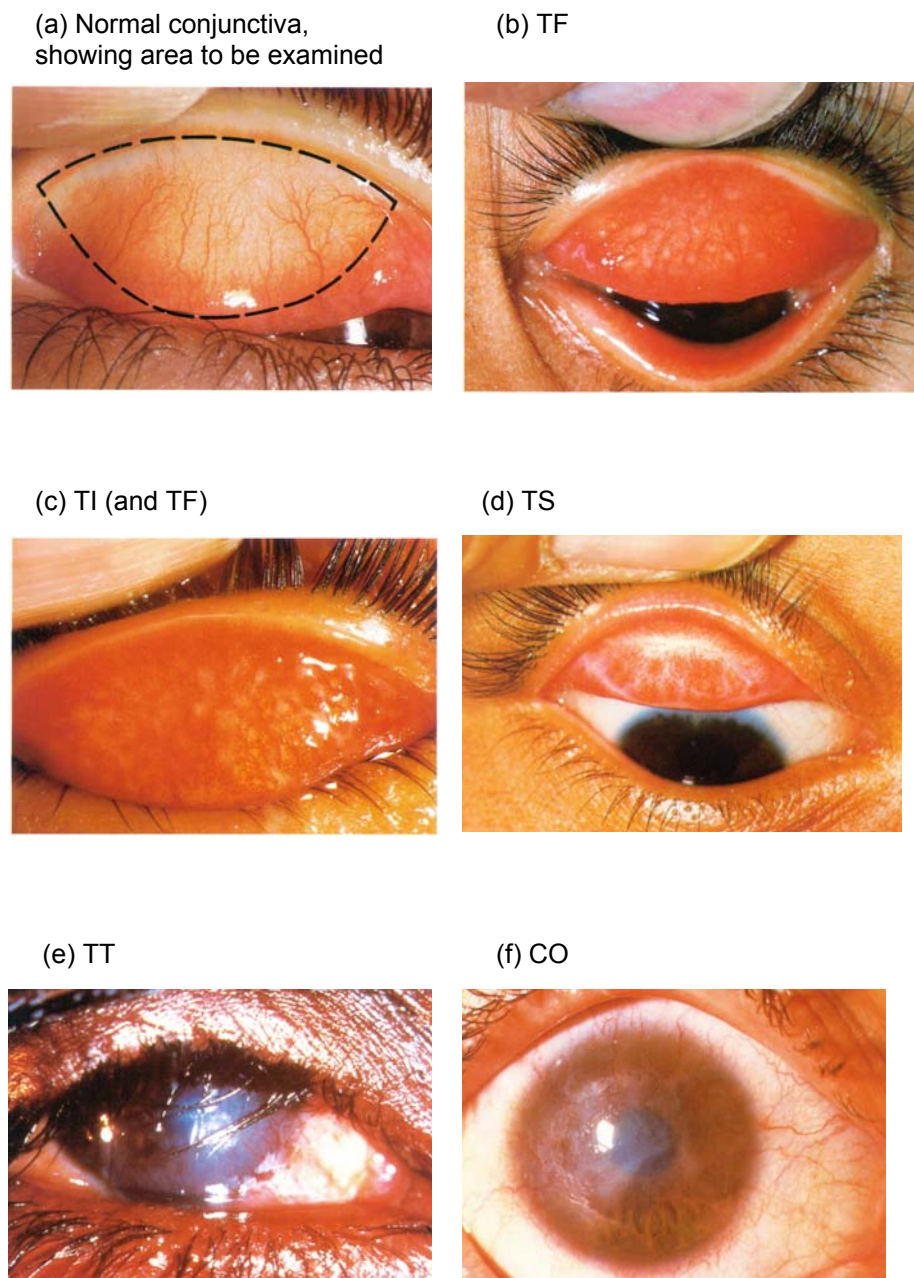
**(ii) Thylefors, Dawson, Jones, West and Taylor, 1987 ('the WHO simplified system')**

The WHO simplified system [200] was designed as a cut-down version of the FPC system, with which it was intended to co-exist. Thylefors *et al.* considered the simplified scheme suitable for use by “less experienced observers” in “population based surveys or for the simple assessment of the disease at the community level” [200](p.480). It provides considerably less information than the FPC scale. However, the simplified system has enjoyed broad-based acceptance, and is now widely used in research, community assessment and programme monitoring by both non-specialists and ophthalmologists alike. It was used in the studies described later in this thesis, and is therefore discussed in some detail here.

The system requires the examiner to assess an individual for the presence or absence of each of five signs (Figure 2.3, p.44):

- TF Trachomatous inflammation – follicular: the presence of five or more follicles at least 0.5mm in diameter, in the central part of the upper tarsal conjunctiva
- TI Trachomatous inflammation – intense: pronounced inflammatory thickening of the upper tarsal conjunctiva obscuring more than half the normal deep tarsal vessels
- TS Trachomatous conjunctival scarring: the presence of easily visible scars in the tarsal conjunctiva
- TT Trachomatous trichiasis: at least one eyelash rubs on the eyeball, or evidence of recent removal of in-turned eyelashes
- CO Corneal opacity: easily visible corneal opacity over the pupil, so dense that at least part of the pupil margin is blurred when viewed through the opacity

Figure 2.3: the WHO simplified system (photos from WHO trachoma grading card)



Preliminary testing of this system by its developers (after trialing and modifying a prototype) involved four observers each examining 179 cases [200]. The inter-observer agreement measurements found in this study are presented in Table 2.2, as they appeared in the original paper.

Table 2.2: Inter-observer agreement for the WHO simplified system: ‘Second study’ from Thylefors *et al.* (1987) [200](p.479)

Sign	Average K-statistic <sup>xi</sup>	K-statistic “range”
TF	0.70	0.66 – 0.74
TI	0.45	0.38 – 0.49
TS	0.75	0.67 – 0.79
TT	0.76	0.68 – 0.81
CO	0.68	0.48 – 0.71

Improved inter-observer agreement was reported from further trials of the system in Tanzania [209]. This trial involved comparisons between an experienced ophthalmologist who had participated in the original development of the system, and two ophthalmic nurses and an ophthalmologist trained by that individual. Two separate studies were performed to assess inter-observer agreement. In the first, 25 eyes were examined by each of four observers, and the scores of the three others compared to those of the instructor. In the second, a single ophthalmic nurse and the instructor evaluated TF, TI and TS only, in 20 eyes. Results are shown in Table 2.3 and Table 2.4.

Table 2.3: Interobserver agreement for the WHO simplified system: ‘First trial’ from Taylor *et al.* (1987) [209](p.486), following 3 hours training, 25 eyes

Sign	Grader			Average K-statistic
	2	3	4	
TF	0.80	0.91	0.60	0.77
TI	0.66	0.71	0.88	0.78
TS	0.73	0.39	0.27	0.46
TT	1.00	1.00	1.00	1.00
CO	1.00	0.63	0.83	0.82

Table 2.4: Interobserver agreement for the WHO simplified system: ‘Second trial’ from Taylor *et al.* (1987) [209](p.487) following several hours further training, 20 eyes

Sign	K-statistic
TF	0.79
TI	0.95
TS	0.87

It is notable from these data that good agreement is not guaranteed even when the observers under comparison are all qualified, experienced personnel trained by the same

<sup>xi</sup> The kappa (K) statistic is a measure of observer reliability for categorical data. It estimates the extent of agreement not due to chance between two sets of observations of the same variable. Kappa has possible values between –1 and +1, with –1 indicating complete disagreement, and +1 complete agreement. Landis and Koch set arbitrary divisions for describing the relative strength of agreement associated with this measurement, as follows: <0.00 poor, 0.00-0.20 slight, 0.21-0.40 fair, 0.41-0.60 moderate, 0.61-0.80 substantial, 0.81-1.00 almost perfect [208].

teacher. The reliability of examiners with variable amounts of training, experience and enthusiasm, operating under different conditions and at different times, or even of the same observer over time, has not been determined. This is not unique to the WHO simplified system [210], and has been a long-standing problem for evaluating trachoma control interventions [211]. In fact, diagnostic reproducibility has been rigorously proven for few signs in clinical medicine.

### 2.5.5 Comparability of grading schemes

The WHO simplified and FPC systems are often said to be directly comparable, allowing derivation of simplified system grades from FPC grades without separate assessment of patients. In the original paper describing the simplified system, Thylefors *et al.* included a table comparing it with Dawson *et al.*'s FPC scheme (Table 2.5).

Table 2.5: "Comparison of the simple grading of trachoma with the grading used in a more detailed system" from Thylefors *et al.* (1987) [200](p.480)

Simple system [200]	Detailed system [132]	Implication
TF	F <sub>2</sub> or F <sub>3</sub>	Presence of inflammatory trachoma
TI	P <sub>3</sub>	Severe intensity of inflammation
TS	C <sub>1</sub> , C <sub>2</sub> or C <sub>3</sub>	Presence of cicatricial trachoma
TT	T/E <sub>2</sub> or T/E <sub>3</sub>	Potentially disabling lesion
CO	CC <sub>2</sub> or CC <sub>3</sub>	Visually disabling lesion

This comparison is not strictly correct: the systems are not directly comparable, though the discrepancies are relatively minor. A diagnosis of TF requires five or more follicles in the central part of the upper tarsal plate, while F<sub>2</sub> is defined as more than five follicles (i.e. six or more) in zones 2 and 3 together: the boundaries 'TF absent' v 'TF present' and F<sub>1</sub> v F<sub>2</sub> do not coincide. Additionally, the woolliness of the definitions of conjunctival scarring in both systems make comparison of grades for this sign problematic.

## 2.6 LABORATORY DIAGNOSIS

In general, the diagnosis of trachoma is made on clinical grounds. Laboratory testing is typically unavailable or unaffordable for clinical care in trachoma-endemic areas. Furthermore, the antibiotics used against active disease can usually be provided at low or no cost to the patient, and are well tolerated by both children and adults,

making presumptive treatment for suspected chlamydial infection a logical therapeutic approach.

For research purposes, however, confirmation of the presence of *C. trachomatis* infection may be necessary, for reasons that will be discussed later. Available assays include microscopy of conjunctival scrapings, isolation in cell culture, direct fluorescent antibody, enzyme immunoassay, serology, nucleic acid hybridization probes, and the nucleic acid amplification tests.

### **2.6.1 Microscopy**

Examination of stained conjunctival scrapings for *C. trachomatis* inclusion bodies is the oldest method for detection of ocular infection. With Giemsa, the stain used by Halberstaedter and von Prowazek [33] and the one still most commonly used [212-216] until microscopy was superseded by superior diagnostic techniques, mature inclusions appear as dark purple masses in the cytoplasm of epithelial cells. Iodine and acridine orange are alternative stains [197]. Gram staining, however, is unreliable: the reaction is negative or variable [44]. Microscopy requires trained technicians [212], is time consuming, and is probably the least sensitive method for diagnosis [217]. Additionally, collection of conjunctival scrapings with a metal blade is painful and unpopular [218]. The availability of less traumatic, more rapid and more sensitive tests has displaced microscopy of scrapings to the sidelines of chlamydia diagnostics.

### **2.6.2 Cell culture**

Chlamydiae are fastidious organisms. Successful culture relies on the use of rich transport media (2-SP, M4) and strict maintenance of the cold chain during transport. In the laboratory, clinical specimens are inoculated onto McCoy cells [103, 146, 171, 217-228], HeLa 229 cells [66, 69, 103, 229-231], or L434 mouse fibroblasts [232]. Usually the cell layer is irradiated [197, 219], or pre-treated with cycloheximide [103, 146, 171, 217, 218, 220, 222-228], diethylaminoethyl dextran [66, 221, 229, 231, 233, 234], or mitomycin C [232] to enhance growth of the organism. The specimen is centrifuged onto the monolayer to aid cellular infection, and the culture is incubated for two to three days. Determining whether the culture is positive or negative requires staining with iodine or Giemsa, or the use of labelled poly- or mono-clonal antibody [235]. One or more 'blind passages' (in which apparently negative cultures are homogenised and inoculated onto fresh monolayers) are usually performed to ensure



that low-level infection is not overlooked. Identification of one inclusion is sufficient to record a positive result [232].

Despite the high specificity of isolation, there are a number of problems associated with using it as a diagnostic test. Inhibition of chlamydial growth in culture can, in theory, be caused by cytokines or antibodies produced by infected tissues and introduced into the culture medium with the clinical sample [236, 237]. Even when using purified elementary body stock which (by definition) should be free of inhibitors, data suggest that only about one EB in 200 is capable of successfully infecting tissue culture cells [238]. Because the protocol for culture is complicated, there are multiple opportunities for variation in factors that impact upon isolation efficiency and, therefore, on sensitivity [239]. This makes it difficult to compare results between laboratories, or even to compare one run to the next in the same laboratory. Additionally, chlamydial culture is expensive, time-consuming, and requires specialised expertise.

Though considered the gold standard for laboratory diagnosis and still legally required to prove chlamydial infection in UK courts [232], it is now accepted that isolation of *C. trachomatis* in cell culture is less than 100% sensitive [217, 227, 231, 232, 239, 240].

### **2.6.3 Direct fluorescent antibody (DFA)**

Immunofluorescence is a technique for detecting cellular molecules. Reagents labelled with fluorescent dye that bind specifically to target proteins are used. A number of different immunofluorescence techniques are possible, depending on whether sample antibody or antigen is the target molecule, and on whether the fluorescent dye is attached to the reagent that binds to the sample ('direct immunofluorescence') or attached to the reagent that binds to an intermediate reagent that binds to the sample ('indirect immunofluorescence'). A direct fluorescent antibody (DFA) test, the Syva MicroTrak<sup>®</sup> (Syva, Palo Alto, CA, USA) was the first diagnostic instrument that used *monoclonal* antibody against *C. trachomatis*, and began the move away from culture to techniques that do not rely on chlamydial viability [241]. The MicroTrak uses labelled antibody to detect a species-specific epitope in MOMP.

For the clinician, specimen collection for DFA is straightforward: conjunctival cells and exudates from a swab are smeared onto a slide in the field or clinic, fixed with methanol [217], and air dried. The slides are easy to ship and store [218]. In the

laboratory, the fixed sample is stained with fluorescein-isothiocyanate-conjugated monoclonal antibody, and examined under a fluorescence microscope. Chlamydial inclusions are highlighted apple-green against the red background of conjunctival epithelial cells. Performing an epithelial cell count provides a straightforward method for determining the adequacy of the collected sample [242]. Samples taken from the upper tarsal conjunctiva yield a greater concentration of infected cells than those from the lower fornix [243].

The MicroTrak performed extremely well in initial sensitivity and specificity trials [244], and the fact that specimens could be transported at ambient temperature to the laboratory gave it a considerable advantage over tissue culture. As a result, the MicroTrak has been widely used in trachoma research studies [103, 146, 168, 181, 217, 222, 223, 226, 245-247]. Comparison between studies is made difficult by the fact that the threshold for defining a positive slide varies from one group of investigators to the next. The major practical disadvantages with DFA are the reliance on the competence of the microscopist, and the length of time required for screening, which averages more than twenty minutes per slide [242].

Biological issues probably also affect the reliability of DFA. Taylor *et al.*, defining a positive slide as one in which five or more EBs were seen, found a discordance of 10% within pairs of replicate DFA ocular specimens taken from the same individuals five minutes apart [242]. This was attributed to sampling variation. It was felt unlikely that this result was due to inadequate specimen collection in one or both of the samples, since all the samples comprising the discordant pairs had satisfactory conjunctival epithelial cell counts. Rather, discordance seemed to occur when a low chlamydial load was present. Discordance within pairs of samples taken two to eight days apart was 22%, suggesting a degree of short-term biological variation in addition to the difference attributable to sampling [242].

#### **2.6.4 Enzyme immunoassay (EIA)**

‘Immunoassays’ include processes that test for specific antigens, and those that test for specific antibodies. Enzyme-linked immunosorbent assays (ELISA), also known as enzyme immunoassays (EIA), are designed to detect antigens or antibodies by producing an enzyme-triggered colour change. For *C. trachomatis*, EIA usually refers to an antigen detection test, using synthetic antibody to detect chlamydial antigen contained in the specimen. The Abbott Chlamydiazyme<sup>®</sup> kit (Abbott Laboratories,

North Chicago, IL, USA) [181, 217, 225, 248] was the first EIA to be produced for commercial release [249].

In the Chlamydiazyme, chlamydial antigens are eluted from collected swabs in specimen dilution buffer. An aliquot of sample eluate is placed in the well of a plastic plate with a polystyrene bead, which adsorbs chlamydial antigens. Polyclonal rabbit anti-chlamydial antibodies are added [250, 251]. Chlamydial antigens reacting with these antibodies include epitopes of LPS and MOMP [252]. A solution of horseradish peroxidase-conjugated goat antibody to rabbit immunoglobulin is then introduced [251]. Following a washing step to remove unbound components, a colourless substrate that is transformed by peroxidase to a coloured product is added to the well. The presence of chlamydial antigen-antibody complexes is demonstrated by measuring the colour change with a spectrophotometer.

The Chlamydiazyme does not require immediate refrigeration of clinical specimens following collection [253], and specimen processing can be completed in four hours [249]. High throughput can be achieved. If processing is delayed, however, prolonged sample storage can reduce the sensitivity of the test [242]. Specificity is also a problem. Chlamydial LPS has at least two antigenic sites, one of which is also found on the LPS of other bacterial species [254]. As a result, *Staphylococcus aureus*, *Haemophilus aegyptius*, *Klebsiella pneumoniae*, *Gardnerella vaginalis*, *Neisseria gonorrhoeae*, *Escherichia coli*, *Streptococcus agalactiae*, *Moraxella lacunata*, *Chlamydia psittaci*, the *Salmonella minnesota* Re mutant, *Acinetobacter lwoffii* and *Acinetobacter calcoaceticus* var. *anitratus*, can all react in the Chlamydiazyme test [217, 250, 255, 256]. Conjunctival infection or sample contamination with any of these organisms could therefore produce a false positive result. A confirmatory assay that selectively blocks binding of the chlamydia-specific epitope can be used to separate true positives from false positives, and thereby increase the specificity of the test [254].

Several commercial EIA alternatives to the Abbot Chlamydiazyme are available, including the Syva MicroTrak EIA<sup>®</sup> (Syva, Palo Alto, CA, USA), and the Pathfinder EIA<sup>®</sup> (Sanofi Diagnostics Pasteur (formerly Kallestad Diagnostics), Chaska, MN, USA).

The Boots CellTech (later Dako) IDEIA<sup>®</sup> (Boots CellTech, Slough, England) [171, 182, 224, 253, 257] incorporated a detection system with the potential for improved specificity, using murine *monoclonal* antibody to chlamydial lipopolysaccharide (LPS) in place of the polyclonal antibody used in the other tests [249, 252]. The detection

principles were later further altered by attaching multiple copies of an anti-LPS monoclonal antibody-alkaline phosphatase complex to a dextran backbone. In this format, designated 'polymer conjugate enhancement' or PCE, each copy of LPS in the sample is able to capture multiple copies of the enzyme, resulting in dual amplification of the signal [258].

A number of rapid 'point-of-care' tests using the EIA format are also available, including Clearview Chlamydia<sup>®</sup> (Unipath, Nepean, Ontario, Canada) and the Quidel QuickVue<sup>®</sup> (Quidel, San Diego, USA). The consensus on these tests seems to be that they sacrifice sensitivity for speed [249, 259, 260].

### 2.6.5 Serology

The first serological test used for diagnosis was a complement fixation (CF) test that detected serum antibodies against the polysaccharide antigens of LPS. Because these epitopes are common to all chlamydial species [91], the specificity of the test was low [261]. Additionally, it had low sensitivity for ocular infections [213].

The microimmunofluorescence (MIF) technique developed by Wang and Grayston [262] was the first method used to classify strains of *Chlamydia trachomatis* into serovars. The serovar-specific antigens delineated by this test can be used in an indirect fluorescent antibody assay to detect anti-chlamydial antibodies in serum or tears, with greater sensitivity than achieved with CF [261]. Serial dilutions of the sample are placed on glass slides to which antigens of different *C. trachomatis* serovars have been fixed (Individual Antigen Serovar Kit, Washington Research Foundation, Seattle, WA, USA). Following incubation, the slides are probed with fluorescein-labelled anti-human immunoglobulin. Testing for the presence of IgA, IgG and IgM can be performed separately [242, 263].

Detecting antichlamydial antibody in serum is difficult, subjective and tedious, and has poor specificity and poor reproducibility. The potential advantage of distinguishing between acute, subacute and chronic infection is not borne out, even with the use of paired acute and convalescent sera [261, 264], because production of IgM antibody is not stimulated by ocular reinfection with a previously encountered *C. trachomatis* serotype [261]. Its uses are limited. Tear MIF has better correlation with clinical trachoma, but suffers from the same practical disadvantages [242].

### 2.6.6 Direct hybridization probe tests

Early attempts to use direct nucleic acid hybridization for the diagnosis of chlamydial infection used radiolabelled *C. trachomatis* DNA and autoradiography, which required an exposure time of thirty-six hours or more. It was successfully used to detect infected cells from tissue cultures, ocular swabs and cervical smears [221, 222]. Unfortunately, sensitivity was thought to be lower than culture [221]. Commercial applications of the technique incorporate significant improvements.

The Gen-Probe PACE 2<sup>®</sup> (Gen-Probe, San Diego, CA, USA) is a nucleic acid probe (NAP), or hybridization probe test. The probe is a synthetic single-stranded DNA molecule complementary to a region of chlamydial ribosomal RNA (rRNA). The sample is heated so that cells are lysed and rRNA released. The probe, labelled with an acridinium ester (AE), is added; it forms a stable DNA:RNA hybrid with its target sequence. Detection is performed with a 'hybridization protection assay': following binding, 'selection reagent' is added, which hydrolyses the AE on unhybridized probes and thereby deactivates it; the AE on hybridized probes is protected within the double helix of the DNA:RNA complex. With subsequent addition of hydrogen peroxide, bound acridinium ester releases a pulse of light that can be detected with a luminometer. Because there are about 2000 copies of rRNA per chlamydial cell, high sensitivity is achieved without amplification of nucleic acid [265, 266].

A second hybridization probe test, the Hybrid Capture II<sup>®</sup> (HCII, Digene, Gaithersburg, MD, USA), uses an EIA to achieve signal amplification, and is therefore classed as a nucleic acid probe/signal amplification assay, or NAPSA. Alkali is added to the clinical sample to lyse cells and denature double-stranded DNA (separate it into its two component strands). An RNA hybridization probe with nucleotide sequence complementary to chlamydial DNA is introduced; because the DNA has been denatured, the probe can anneal to its target region. The RNA:DNA complex is then captured by antibody bound to microwell plates. Antibodies labelled with alkaline phosphatase are added to bind to the bound complex. After washing, the presence or absence of bound DNA can be determined by adding a substrate for alkaline phosphatase: cleavage of the substrate results in emission of light [265, 267]. The test is rapid and reproducible [267], but, for the diagnosis of ocular chlamydial infection, direct hybridization assays have been largely overlooked in favour of the nucleic acid amplification tests discussed below.

### 2.6.7 Polymerase chain reaction (PCR)

The polymerase chain reaction is a technique for amplifying DNA: assays based on it are part of the group of nucleic acid amplification tests, or NAATs. PCR uses the enzyme DNA polymerase. A variant of this enzyme is found in the nucleus of all replicating cells. *In vivo*, its function is to duplicate DNA during the cell's preparation for its own division. In 1983, Mullis realised that exponential growth in the number of copies of a target DNA sequence could be achieved *in vitro* if repeated rounds of DNA polymerase-catalysed duplication were made to occur back-to-back [268, 269].

To undertake PCR, sample is added to a reaction mixture containing an excess of deoxyribonucleotide triphosphates, heat stable DNA polymerase, and two primers. Primers are short, synthetic oligonucleotides that flank the region to be amplified. One primer is complementary to the sense (+)-strand, and one to the antisense (–)-strand at the opposite end of the target sequence.

There are three phases of the reaction. All take place in the same vessel, but at different temperatures. The mixture is first heated to a temperature between 90 and 95°C to denature DNA. At a reduced temperature, the primers anneal to their respective binding regions. The mixture is then again heated to enhance the activity of the DNA polymerase, which extends each chain from the 3' end of its annealed primer to produce two double-stranded copies of the target sequence. Both of the two newly-synthesized primer extension products contain the appropriate primer-binding regions, and, after heat-induced separation from the target, can themselves function as templates – alongside the original templates – in the next round of duplication. Multiple repetitions of the denaturation / annealing / extension process therefore result in exponential accumulation of the target. PCR is now usually automated in a thermal cycler, which rapidly and reliably changes the temperature of the reaction vessel to provide appropriate conditions for each stage of the amplification process.

PCR was originally developed as a method for detecting the changes in the  $\beta$ -globin gene that underlie sickle cell anaemia [269]. Its uses, though, are far more wide-ranging. PCR is ideally suited to the detection of DNA of fastidious and non-cultivable infectious agents, since it does not rely on the presence of viable organism in the sample. The first bacterium for which a PCR-based detection method was published was *Chlamydia trachomatis* [270].

A number of different nucleic acid sequences have been used as targets in PCRs for the detection of *C. trachomatis*. These include the chlamydial cryptic plasmid (pCT)

[105, 225, 231, 257, 271], *omp1* coding for MOMP [225, 234, 270-272], the gene coding for 16s rRNA [225, 231, 273], and *omp2* coding for OmcB [79, 274]. With the exception of pCT, all of these targets are sequences found on the CT chromosome, which includes two complete rRNA operons, and single copies of *omp1* and *omp2*.

PCR directed at plasmid genes [257] or *omp1* [234] is thought to be both sensitive and specific for the diagnosis of *C. trachomatis* infection. Primers designed for the chlamydial rRNA gene amplify this DNA sequence in *C. trachomatis*, *C. psittaci* and *C. pneumoniae* [231], which reduces the specificity of the assay. The *omp2*-based PCR has also been found to return positive results on samples containing any of these three species; subsequent restriction endonuclease digestion and gel electrophoresis permits species and strain identification of isolates [274], but this assay has not been extensively utilised in published studies to date.

Mahoney *et al.* [225] estimated that plasmid-based PCRs are between 10 and 10,000 times more sensitive than PCRs directed against chromosomal genes. This is probably at least partly attributable to the presence of multiple copies of the plasmid per chlamydial cell [225, 257, 271]. Bailey *et al.* [206] also suggested that using a plasmid target gives greater sensitivity. Using serial dilutions of DNA standards, they calculated the lower detection limit of their plasmid-based PCR to be 1 to 10 EBs, compared with 10 to 100 EBs for a PCR against *omp1*.

A commercially produced PCR kit, Amplicor Chlamydia<sup>®</sup> (Roche Diagnostic Systems, Branchburg, NJ, USA) targets a 207 base-pair sequence within pCT. An aliquot of prepared sample is added to the PCR master mix, which contains heat stable polymerase, an internal control (see below), two biotinylated primers, cofactors, deoxynucleotide triphosphates (dNTP), and the enzyme AmpErase<sup>®</sup> (uracil-N-glycosylase). The dNTP deoxyuridine triphosphate (dUTP) is included in place of deoxythymidine triphosphate (dTTP). Together, the substitution of thymidine by deoxyuridine in the reaction mixture (and therefore, ultimately, in the amplified product) and the addition of AmpErase help to prevent DNA produced during PCR from contaminating subsequent runs [275]. AmpErase degrades DNA containing deoxyuridine by breaking the deoxyribose chain at the C<sub>1</sub> position. Naturally-occurring DNA does not contain deoxyuridine, and is therefore not a substrate for the enzyme. As the reaction mixture is heated in the first thermal cycling step, any contaminating product DNA molecules are cut into short oligonucleotides by breakages induced at each deoxyuridine residue, rendering them non-amplifiable. AmpErase becomes

inactive above 55°C, and, because the reaction vessel is held above this temperature for the duration of the amplification process, the target amplicon is not affected. Following amplification, before the reaction mixture has a chance to cool, AmpErase is destroyed by immediate addition of 'denaturation solution' [276].

Denaturation after cycling also separates the double-stranded amplified product into single strands of DNA. An aliquot of the reaction solution is placed in a microwell to which oligonucleotide probes complementary to the pCT target sequence have been bound. Specific amplified product hybridizes to the probes, and the microwell plate is washed. Avidin horseradish peroxidase conjugate is then added, which binds to any amplicon that has been captured in the microwell. After another washing step, hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) are added; bound horseradish peroxidase catalyses the formation of a coloured compound from the TMB. Optical density (OD) can be read with a photometer [276].

Reproducibility problems have been reported in a very small proportion of samples tested with the Amplicor assay, with results obtained from multiple aliquots of the same specimen yielding ODs ranging from negative (OD<0.200) to the upper positive limit of the photometer (OD>3), even when tested in the manufacturer's own laboratories [277]. Similarly, the result obtained from a PCR using primers for pCT does not invariably correspond to that from a PCR using primers for *omp1* [277]. Possible explanations include contamination of PCR tubes, contamination of the microwell plates used in the detection step, non-specific binding of primers during amplification, non-specific hybridization during detection, a very low concentration of organism, technical errors, or the presence of inhibitors that undergo time-dependent inactivation [277-279]. Investigation of the reasons for these inconsistencies is still ongoing [280].

The possible presence of inhibitors to DNA amplification within samples needs to be kept in mind. They can be found in specimens obtained from the conjunctiva and from the urogenital tract [168, 226, 281]. Inhibition may in some instances be overcome by prolonged storage prior to processing, which presumably allows time for degradation of the inhibitory factor [226, 278, 282], sample dilution [168, 226, 279, 281], freezing at -70°C [279], or heat treatment at 95°C for 10 minutes [281]. The Amplicor assay gives the user the additional option of monitoring each individual test for inhibition by including an internal control (IC) in each amplification reaction. The IC is a synthetic nucleic acid sequence with primer binding regions identical to those of the test-specific target, and a randomised internal sequence that has similar length and



base composition to the target. As a result, the IC and the target region of the plasmid from the clinical specimen are co-amplified with equivalent amplification efficiency. The probe binding region of the IC is unique, allowing reliable differentiation from plasmid DNA [276]. Post-amplification, detection of the target and IC are undertaken separately. A positive test for amplification of the IC indicates that PCR was not inhibited, and therefore that inhibition was unlikely to have produced a false negative result.

The nature of PCR, in which small quantities of DNA are exponentially multiplied, makes it inherently vulnerable to spuriously positive results following contamination of samples during collection, preparation and processing. Airborne target DNA in the laboratory is a particular danger [257]. Use of separate laboratory zones for sample preparation, amplification and detection; restriction of sample handling to negative pressure safety cabinets; stringent adherence to procedures for decontamination; and inclusion of negative controls all help to protect (and demonstrate protection of) the integrity of results. Similar precautions must also be taken to safeguard reliability of the other nucleic acid amplification tests, described below.

Roche also produce an automated version of the Amplicor system, known as the Cobas Amplicor<sup>®</sup>. For urogenital swabs and urine, the two formats appear to have comparable sensitivity and specificity [283].

#### **2.6.8 Ligase chain reaction (LCR)**

DNA ligase is an enzyme that **inks** (ligates) fragments of DNA, by inducing the formation of phosphodiester bonds between the 5' phosphate of the first fragment and the 3' hydroxyl of the second. Its function is to repair 'nicks' in the phosphodiester backbone of double-stranded DNA, so will only catalyse the linkage of DNA fragments that are annealed adjacent to each other opposite their complementary sequences. LCR uses this enzyme as part of an iterative process of denaturation, annealing and ligation.

Prepared specimens are added to a reaction mixture containing heat stable ligase, and two *pairs* of labelled oligonucleotide probes. One probe of each pair is labelled with a capture hapten, and one with a detection hapten. Sample DNA is denatured by heat. The two probes in each pair hybridize adjacent to each other, one pair binding to the sense strand, and the other to the antisense strand of the denatured target. DNA ligase seals the nick within each pair of juxtaposed probes, forming products that can serve as targets for the next round of the reaction. Repeated cycling effectively results

in amplification of covalently-linked DNA probe pairs dually-labelled with both capture and detection haptens, in subtle contrast to the target amplification of PCR [284].

In gapped LCR, DNA ligase functions alongside DNA polymerase in a process that combines LCR with PCR. Each pair of probes is designed to anneal to adjacent regions on the same strand leaving a gap of a few nucleotides. This gap is filled by DNA polymerase. DNA ligase then joins the 3' probe of each pair to the intervening nucleotide sequence [285].

The Abbott LCx<sup>®</sup> (Abbott Laboratories, Abbott Park, IL, USA) targets a sequence of pCT [286] in a gapped LCR that is now widely used for laboratory diagnosis of genital and ocular infection. Its detection step employs microparticle-bound antibody to the capture hapten, which is then collected in a glass fibre matrix. Unattached detection hapten is washed away. The presence of attached detection hapten is revealed through the use of an enzyme immunoassay [284]. Because DNA ligase is inhibited by even a one base-pair mismatch near the inner ends of the probes (at the site of the nick), high specificity is obtained without the use of an oligonucleotide hybridization probe in the detection protocol [287]. Unfortunately, LCx lacks an internal control, so there is no inbuilt check for inhibition of amplification.

### **2.6.9 Strand displacement assay (SDA)**

The strand displacement assay was developed as an alternative to PCR and LCR for amplifying target DNA sequences. Fundamentally, SDA is a variant of the polymerase chain reaction. It uses a restriction endonuclease to create a nick in one strand of double-stranded DNA, and an exonuclease-deficient form of the *Escherichia coli* DNA polymerase I (or equivalent) to extend from the site of the nick whilst displacing intact the downstream complement of the target [288]. Because repeated denaturation is not required, the reaction can proceed isothermally.

The BDProbeTec<sup>®</sup>ET (Becton Dickinson, Franklin Lakes, NJ, USA) is a commercially-produced SDA that targets *C. trachomatis* plasmid DNA. It incorporates a real-time fluorescence detection system, allowing amplification and detection to occur in the same sealed microwell. This reduces specimen handling, and minimises the potential for cross-contamination through release of amplified DNA from the reaction tube [289, 290]. The assay is rapid, simple, and requires little in the way of hands-on time from laboratory staff. According to the manufacturer, as few as ten to fifteen *C. trachomatis* EBs can be reliably detected [289]. No published studies have used either

SDA or the next assay discussed, transcription mediated amplification, for identifying chlamydiae in ocular swabs.

#### **2.6.10 Transcription mediated amplification (TMA)**

Transcription mediated amplification mimics the RNA replication strategy of retroviruses, producing an RNA amplicon by means of complementary DNA (cDNA) intermediates [291]. It can be used against any type of nucleic acid target. The commercial assay developed for detection of *C. trachomatis*, the Gen-Probe AMP-CT (Gen-Probe, San Diego, CA, USA), is directed against ribosomal RNA. Reverse transcriptase (RT) and RNA polymerase are used to achieve amplification. A primer binds to target rRNA, allowing RT to make a DNA copy by extending the primer from its 3' end. The rRNA of the DNA:rRNA hybrid is degraded by the RNAase-H activity of RT. A second primer binds to the cDNA, and RT extends it to complete a double-stranded DNA molecule. The first primer includes a promoter sequence for RNA polymerase; this enzyme now recognises that promoter and transcribes 100-1000 copies of RNA, each of which can serve as template for RNA-dependent DNA synthesis catalysed by RT. Like the SDA, TMA can be carried out without thermal cycling. Detection is performed using the same hybridization protection assay technique described earlier for the Gen-Probe PACE 2 [266].

#### **2.6.11 Quantitative PCR**

Culture, DFA and the first generation of EIAs can be optimised to function as semi-quantitative techniques. Unfortunately, these tests lack sensitivity. The nucleic acid amplification tests discussed so far (which are highly sensitive) are purely qualitative. The difficulty with using nucleic acid amplification for quantification is the exponential nature of DNA reproduction: small changes in amplification efficiency will produce a large change in the amount of DNA produced [292]. In other words, the amount of DNA present at the end of forty PCR cycles may depend as much on minor variations in reagent concentrations, the properties of any contaminating DNA, or the presence of inhibitors, as it does on the amount of DNA present in the original sample. Small between-tube variations on the same run may be caused by temperature differences along the heating block, and other factors that are difficult to characterise. In addition, as will be explained below, PCR amplification efficiency changes as the reaction

proceeds. Reliable quantification of a sample can therefore not be achieved by comparing end-PCR yield with that of an external control [292].

In each PCR cycle ( $i$ ), the number of copies of product at the end of the extension step ( $P_i$ ) is determined by the number present at the end of the previous cycle ( $P_{i-1}$ ) and the efficiency of DNA synthesis in that cycle ( $E_i$ : a number between 0 and 1) [293].

$$P_i = P_{i-1} + (P_{i-1} \times E_i) \quad (1)$$

An alternative way of expressing this is

$$P_i = P_{i-1} \times (1 + E_i) \quad (2)$$

The amount of product after the first cycle is therefore given by

$$P_1 = P_0 \times (1 + E_1) \quad (3)$$

and after two cycles by

$$P_2 = P_1 \times (1 + E_2) \quad (4)$$

Substituting equation (3) into (4):

$$P_2 = P_0 \times (1 + E_1)(1 + E_2) \quad (5)$$

After  $n$  cycles, the amount of product present will be given by

$$P_n = P_0 \times \left( \prod_{i=1}^n (1 + E_i) \right) \quad (6)$$

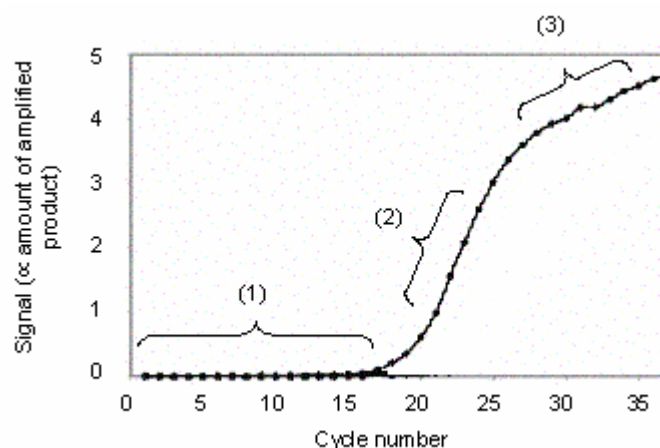
where

$$\prod_{i=1}^n (1 + E_i) = (1 + E_1)(1 + E_2)(1 + E_3) \dots (1 + E_n) \quad (7)$$

$P_n$  can be determined with a spectrophotometer once thermal cycling is complete, but this alone is unhelpful in determining  $P_0$ , because the values of  $E$  are not known. This problem can be solved in one of two ways. In relative quantification (co-amplification, or end point quantitative PCR), a known amount of unrelated DNA (standard) is co-amplified with the sample (target) in the same reaction tube, and the amount of both the standard and target sequences measured at the conclusion of the reaction. Assuming that the amplification efficiencies  $E[S]$  (for the standard) and  $E[T]$  (target) are the same in each cycle<sup>xii</sup>, the ratio of the amounts of the two sequences after PCR will reflect their starting ratio [293].

The alternative solution is kinetic quantitative PCR. A PCR can be considered to have three phases: (1) an early background phase, in which the background signal (often, for example, fluorescence) from the detection system is greater than the signal generated by the amplified product; (2) an exponential phase; and (3) a plateau phase, when amplification efficiency falls due to a build up in inhibitory reaction products, the concentration of polymerase becoming limiting, and product renaturation competing with primer binding during annealing [294].

Figure 2.4: PCR profile. The signal (shown on the vertical axis) is related to the amount of amplified product, and in this example has been acquired once per cycle. (1)=background phase, (2)=exponential phase, (3)=plateau phase (modified from Rasmussen (2001) [294]).



<sup>xii</sup> The efficiency of the reactions can change over the course of the amplification period, provided  $E[s]$  and  $E[t]$  change synchronously [293].

Kinetic quantitative PCR assumes that  $E$  is constant in each cycle. Because this is only true during the exponential phase, the background and saturation phases of the reaction must be disregarded. If  $E$  is constant, equation (6) becomes

$$P_n = P_0 \times (1 + E)^n \quad (8)$$

If  $j$  is the number of cycles that elapse between two successive measurements of  $P$ ,

$$E = -1 + (P_i / P_{i-j})^{1/j} \quad (9)$$

To ensure that  $E$  has remained constant up to the point at which the last measurement of product is made,  $E$  must be estimated at least twice. As a result, the number of copies of target present in the reaction vessel must be determined three or more times during amplification. To prevent the underestimation of  $E$ , it is critical that the method used to detect the target gives a signal linearly dependent on the amount of product present, or, if compression of the signal occurs, that this is compensated for. Once the value of  $E$  has been calculated,  $P_0$  can be derived from the measured amount of product by rearranging equation (8) [293]:

$$P_0 = P_n / (1 + E)^n \quad (10)$$

True kinetic (or ‘real-time’) PCR can be achieved by having the reactions take place in closed, optically clear glass vessels, and analysing product generation by detection and quantification of a fluorescent reporter after each extension step. This eliminates the need for repeated tube opening and post-PCR processing, improves accuracy, reduces the risk of sample contamination, and enhances automation [293, 295]. The LightCycler (LC, Roche Molecular Systems, Branchburg, NJ, USA) has been designed using these principles. It uses glass capillaries as reaction vessels [296]. Samples are loaded into bulbs at the top of the capillaries and spun into columns at the capillary tips by centrifugation [297]. Prepared capillaries are placed in a circular carousel that rotates within the LightCycler’s cylindrical sample chamber. A heating cartridge and venting fan control the temperature of the chamber. Because the heat capacity of air is low, the volume of the capillaries small and their surface to volume ratio high, cycling can be rapid, the requirement for expensive reagents is minimised,

and the temperature of the reaction mixture is homogeneous. The fluorescent signal, generated by one of a number of alternative reporter systems (see below), is obtained from each capillary during the elongation phase, by sequentially positioning each tube in a beam of photodiode-generated light. Data can be displayed as fluorescence v cycle number (in the manner of Figure 2.4, p.60) in real time for each sample [297].

Three different approaches to fluorescence monitoring have been developed.

(1) Double-stranded DNA (dsDNA) binding dyes include ethidium bromide [298, 299], YO-PRO-1 [300] and SYBR<sup>®</sup> Green I (Molecular Probes, Eugene, OR, USA) [301]. These dyes are intercalators: they bind to double-stranded but not single-stranded DNA. However, because some background fluorescence is detectable even in the absence of dsDNA, and because higher concentrations of dsDNA show proportionally less fluorescence (presumably because the amount of dye is limiting<sup>xiii</sup>) [301], the fluorescence intensity is not linearly proportional to the concentration of dsDNA. As a result, the graph of fluorescence intensity against time or cycle number will not be the same as [dsDNA] against time [299]. Calibration using a graph of fluorescence v [dsDNA], which can be generated using samples containing known copy numbers, is required [299].

Double-stranded DNA dyes bind to all double stranded DNA. This makes them economical, but means that the non-specific products that accumulate after multiple cycles of amplification will result in the generation of a fluorescent signal. Characterisation of the DNA product by gel electrophoresis [297] or melting curve analysis [302, 303] is important to ensure specificity. The latter technique involves continuous monitoring of fluorescence throughout a complete temperature cycle. Denaturation can be observed as a loss of fluorescence as the temperature passes the melting point, which is dependent on the length, G+C/A+T ratio, and sequence of the product [302]. Additionally, modified forms of DNA polymerase, such as HotStarTaq<sup>™</sup> (Qiagen) can be useful for maximising specificity: this enzyme has no activity at ambient temperatures, being activated by a fifteen minute 95°C incubation step prior to the first amplification cycle. As a result, no misprimed products or primer dimers are formed during setup of the PCR [304]. Accurate quantification of low copy number samples is then limited only by stochastic effects [303].

(2) A number of methods rely on release of fluorescence quenching. Dual-labelled hydrolysis probes have two covalently bound fluorophores. Upon irradiation,

one of the fluorophores (e.g. fluorescein [301] or 6-carboxy fluorescein (FAM) [295]) transfers resonance energy to the other (e.g. rhodamine [301] or 6-carboxy-tetramethyl rhodamine (TAMRA) [295]) rather than fluorescing. This is termed ‘quenching’. Probes are designed to anneal to an internal region of the target sequence. Polymerase replicates DNA to which the probe is bound, and its 5'-exonuclease activity [305] cleaves the probe between the points of attachment of the donor (reporter) fluorophore and the quencher. When excited by incident light, the reporter will emit fluorescence, the intensity of which increases in proportion to probe cleavage. No signal is generated in the absence of product [301]. TaqMan<sup>®</sup> probes (Applied Biosystems) use this approach.

Molecular beacons are oligonucleotide probes with reporter and quencher fluorophores bound at either end, and 5'-terminal and 3'-terminal sequences that are complementary to each other. As a result, when the oligonucleotide is free in solution, it assumes a hairpin shape, bringing reporter and quencher dyes into close approximation. This suppresses reporter fluorescence. When the probe binds to the amplified product, it undergoes a conformational change, separating reporter and quencher by sufficient distance to permit fluorescence on irradiation [306].

(3) Two separate specific oligonucleotide probes can also be used. One is labelled with a donor fluorophore, such as fluorescein, and one with an acceptor fluorophore, such as LightCycler Red 640 [307, 308] or Cy5 [297, 301]. The two probes are designed to independently hybridize (in head-to-tail conformation) to adjacent sequences on the amplified product, which results in very high specificity [307]. Incident light excites the donor fluorophore. Excitation energy is transferred to the acceptor, which emits fluorescence. No fluorescent signal is generated in the absence of product [301].

*Omp1* and plasmid sequences of *C. trachomatis* have both been used as targets in quantitative PCR assays developed for the LightCycler. This will be discussed further in Section 7.1.2 (p.176).

#### **2.6.12 Correlation of laboratory tests with clinical signs of trachoma**

Advances in techniques for the laboratory diagnosis of *C. trachomatis* have generally been driven by the requirement for improved detection of urogenital infections. As a result, the bulk of the literature examines their utility in that setting.

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<sup>xiii</sup> If SYBR Green I is used at high concentration (<1:7000 dilution), it inhibits amplification [301].



This analysis considers only the relationship of test positivity with clinical signs of *ocular* disease, and only uses results of studies using either the FPC or WHO simplified grading schemes. Where information from trachoma treatment trials has been included, only baseline (pre-treatment) data are presented. Data are shown in Appendix 1 (p.192).

The data suggest that – even in areas hyperendemic for trachoma – not all individuals with active disease test positive for ocular CT, regardless of the assay employed. Equally, the absence of signs is no guarantee of a negative result. Hypotheses for the poor correlation of laboratory tests with clinical findings fall into three categories: wrong test result; wrong clinical diagnosis; and true finding. Consideration of the last of these possibilities may help us further elucidate the natural history of human ocular chlamydial infection.

#### **(i) Factors influencing the accuracy of the test**

Collecting a conjunctival swab or scrape involves sampling cells. If the intensity of infection is low, the proportion of conjunctival cells that are infected will be low, so the number of chlamydiae collected in a fixed number of sampled cells will have Poisson distribution. Chance (sampling variation) will determine the likelihood that one swab contains sufficient cells with sufficient numbers of EBs and RBs to exceed the test's threshold for returning a positive result.

Unfortunately, it is impossible to collect a fixed number of conjunctival cells. Factors affecting cell yield include the intensity and maturity of infection; the abrasiveness and absorptive ability of the swab; the force with which the swab is applied; the speed, distance travelled and rotation of the swab head; the subject's reaction to the procedure; and the presence of any pathology rendering the conjunctival tissues more friable. The relative contribution of antigen or nucleic acid from free EBs within extracellular fluid is equally difficult to quantify.

Trachoma is usually found in more remote communities of poor countries. Suboptimal storage conditions and delays in shipping or processing can be expected. In these circumstances, deterioration of the sample between collection and testing is possible, and may affect test performance [169, 309].

Occasional technical errors and minor variations in protocol are inevitable, and may affect the reliability of any assay. Inadequate sampling of conjunctival epithelium (particularly in children unwilling to submit to examination), or cross-contamination of samples at the point of collection are both potential problems. In the laboratory, several

tests are particularly operator dependent: microscopy, culture and DFA each demand expertise, patience, and concentration; microscopy and MIF are by nature somewhat subjective.

Billion-fold amplification of chlamydial nucleic acid in the NAATs raises the possibility of cross contamination of samples that are subsequently tested in the same laboratory.

Some substances present in ocular specimens might inhibit chlamydial growth in tissue culture. Some substances present in ocular specimens inhibit DNA or RNA polymerase, DNA ligase, or reverse transcriptase. The Amplicor kit gives the user the option of specifically checking each assay for successful amplification, and strategies (such as preheating the specimen to 95°C) that inactivate some inhibitors of nucleic acid amplification have been empirically determined. Equivalent steps to exclude the presence of growth inhibitors in culture would be more expensive and time-consuming.

Some assays target epitopes (EIA for LPS) or genes (PCR for rRNA or *omp2*) that react with similar molecules in organisms other than *C. trachomatis*. Even when the target is specific, non-specific nucleic acid amplification or non-specific hybridization during detection can theoretically occur, producing false-positive results.

The existence of plasmid-free strains of *C. trachomatis* has already been mentioned (Section 2.1.5(b), p.30). The usefulness of Amplicor and LCx as diagnostic tools depends critically on the fact that these strains are extremely rare.

## **(ii) Factors influencing the accuracy of clinical diagnosis**

Currently accepted thresholds for clinical diagnosis of trachoma exclude some individuals with good evidence of disease [206]. For example, using the WHO simplified system [200], five follicles in the central part of the upper tarsal conjunctiva is labelled ‘active trachoma’, while four follicles (assuming there are no other signs of trachoma) is called ‘normal’<sup>xiv</sup>. In both the WHO simplified system and the FPC system [132], pannus (once considered a *sine qua non* of diagnosis [202, 310]), and follicles seen at the limbus or in the fornices must be ignored.

Conversely, both grading systems force a diagnosis of trachoma on individuals with inflammatory conjunctival disease of other aetiology. As already mentioned, papillae

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<sup>xiv</sup> The presentation by Thylefors *et al.* of “Normal conjunctiva” under the heading “The grading system and its use” [200](p.478) (rather than separately – in the introduction to the system, for example) was unfortunate, since it can be interpreted as indicating that the scheme has six mutually exclusive clinical divisions. In fact, TF, TI, TS, TT and CO can all be seen in the same eye at the same time. Each sign needs to be graded as being present or absent.

are poorly predictive of chlamydial infection when seen in the absence of follicles, or in an area of low trachoma endemicity. It has also been postulated that the characteristic trachomatous follicular reaction may be elicited by non-chlamydial stimuli in individuals with a history of active trachoma [311].

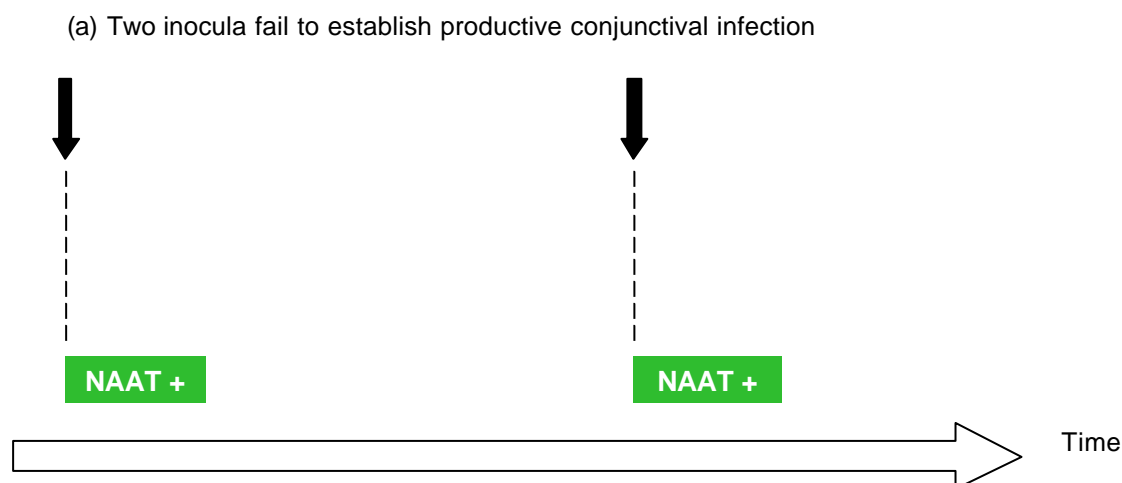
In brief, then, the clinical grading schemes in current use have imperfect sensitivity and specificity. Final judgement of diagnostic tests on the basis of a comparison with the presence or absence of the standardised signs is inappropriate.

### (iii) Factors relating to the natural history of infection

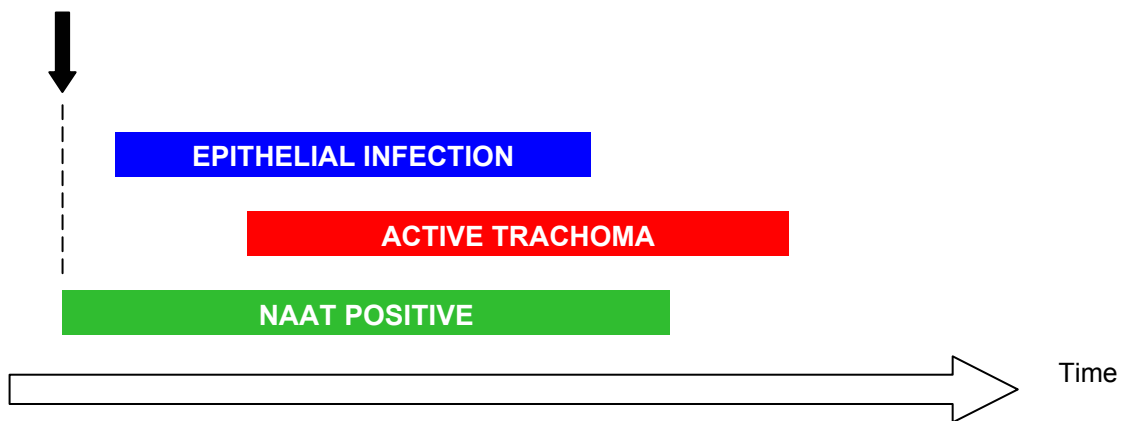
It is conceivable that, in the absence of any clinical signs of disease, some positive tests represent transient contamination of the eye with an inoculum too small to establish infection of epithelial cells (Figure 2.5(a)).

There is good evidence that the development and resolution of signs lags behind the start and finish of the period of laboratory positivity by DFA and EIA [181] (Figure 2.5(b)). A similar offset between laboratory evidence of infection and clinical status has been noted using PCR [206]. In other words, it is possible that active disease may become clinically apparent weeks to months after infection, and that a similar period may elapse between clearance of infection (or cessation of bacterial shedding) and the disappearance of clinical disease [206]. Additionally, ‘persistent’ (Figure 2.5(c)) and ‘cryptic’ (Figure 2.5(d)) *C. trachomatis* infection have both been hypothesised, as mention in Section 2.4.2 (p.35).

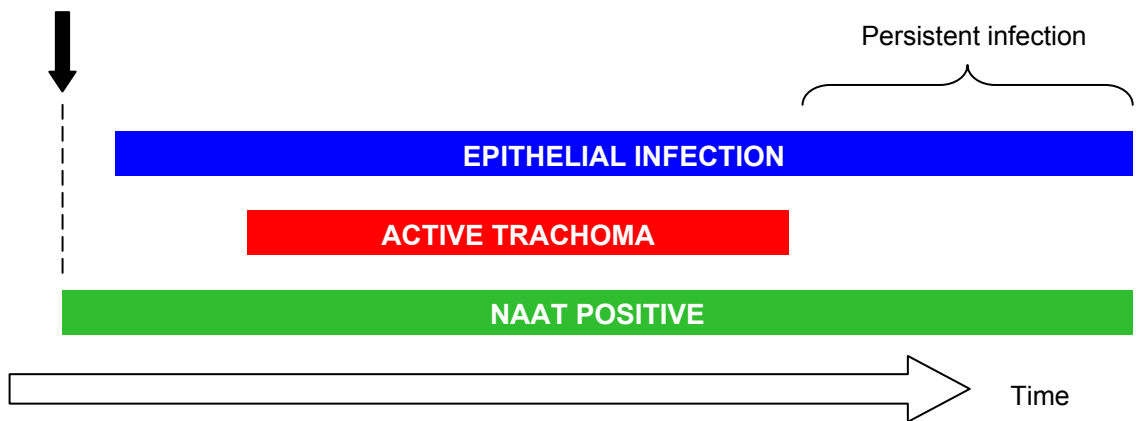
Figure 2.5: Hypothesized models for the relationship between infection, disease, and test positivity following introduction of *C. trachomatis* into the conjunctival sac (↓ = *C. trachomatis* inoculum)



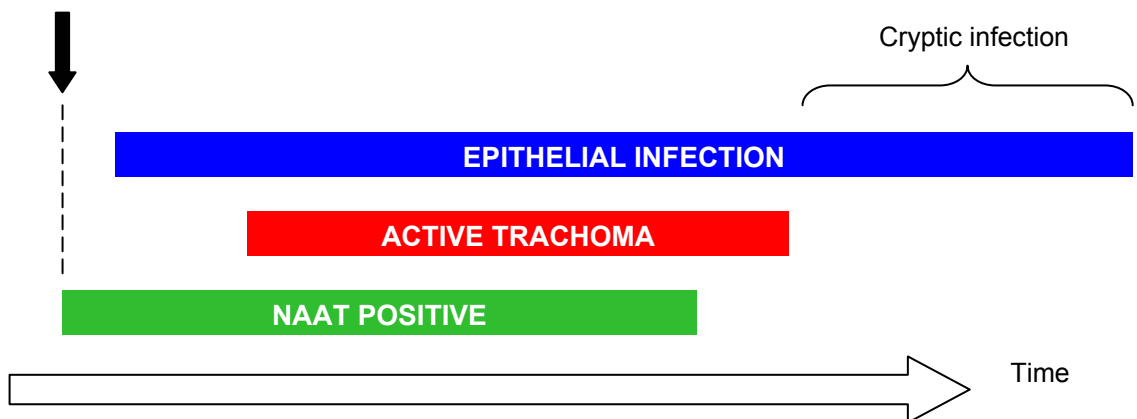
(b) Inoculum establishes productive infection, which results in the appearance of clinical signs of active trachoma



(c) 'Persistent' infection



(d) 'Cryptic' infection



### 2.6.13 Sensitivity and specificity of laboratory tests

Cell culture has long been regarded as the 'gold standard' of *Chlamydia* diagnosis because its specificity is thought to be near-perfect. Its sensitivity is known to be imperfect. True infection status has therefore been impossible to determine, and numerical values assigned to the specificity or sensitivity of diagnostic tests vary

significantly from one estimate to the next. Despite this, such values are routinely quoted.

Newer tests, such as the NAATs, are believed (for biological reasons) to be more sensitive than cell culture. Investigators comparing the performance of these tests against that of culture assume that at least some of the apparent false positives (by the new test) are actually true positives that have been missed by culture. To estimate the performance characteristics of the new test, these apparent false positives are often evaluated using 'discrepant analysis'. In this procedure, gold-standard-negative, new-test-positive samples are further tested by one or more other appeal assays; the return of one or more positive results from these assays labels the sample a true positive. In evaluating the nucleic acid amplification tests for *C. trachomatis*, the appeal tests used have typically been other NAATs, often using a different method of amplification or a different target nucleic acid sequence [312-314].

Hadgu has argued that discrepant analysis is fundamentally flawed, because the restriction of retesting to the apparent false positives must upwardly bias sensitivity and specificity estimates for the new test, regardless of the accuracy of the appeal tests used for resolution [314, 315]. In fact, any test used for resolution, no matter how ridiculous<sup>xv</sup>, can only improve or leave unaltered the calculated sensitivity and specificity of the new test [316]; the magnitude of the bias depends on the underlying prevalence of disease and the independence of the appeal and new tests [312, 316]. This criticism has provoked vigorous and often heated debate [313, 317, 318-324].

Despite the rancour, there is probably broad acceptance that nucleic acid amplification tests represent a significant advance in the diagnosis of chlamydial infections. They appear to be significantly more sensitive than assays available previously, with the potential to detect chlamydiae "beyond clinically relevant levels" [287](p.731). Using dilutions of purified EBs, and assuming the presence of 10 plasmids per organism, Shattock *et al.* estimated the detection limits of the Cobas Amplicor, Amplicor plate kit, and LCx assays to be approximately 1–2, 2–4, and 2 EBs (per tested aliquot) respectively [325]: several orders of magnitude better than the sensitivity estimated by Miyashita and Matsumoto [89] for IDEIA. Additionally, the NAATs seem to be highly specific. Quality control in manufacturing and the skill of the user may now be more likely to affect NAAT accuracy than the underlying biological characteristics of the assay [280].

### 2.6.14 The place of laboratory tests in community assessment

In the Surket district of Nepal in 1997, Baral *et al.* examined all children aged one to ten years from six arbitrarily chosen villages. Forty-six of 726 children (6%) had clinical signs of active disease (TF and/or TI) in the right eye. Swabs were taken from the same eye of all those with active disease and a random selection of one in eight of those without active disease. All samples were negative by an LCR directed against pCT and by an LCR directed against *omp1*. Microscopy disclosed the presence of non-chlamydial bacteria on three of fifteen swabs: gram-negative bacilli consistent with *Moraxella sp.* on two slides, and gram-positive cocci consistent with *Streptococcus pneumoniae* on one. It is possible that these and other organisms are able to reproduce the clinical picture of active trachoma, particularly in those who have previously had a follicular reaction to ocular chlamydial infection [311]. In a separate study in low prevalence areas of Nepal and China, only 8% of clinically active cases were LCR-positive [326]. Additionally, even in hyperendemic areas, the correlation between disease and infection appears to worsen following antibiotic treatment [327].

Using highly sensitive assays for *C. trachomatis*, the lack of evidence of ocular chlamydiae in populations with a low but measurable prevalence of signs of active trachoma raises the question of how elimination of trachoma from an area will be certified. Absence, or a very low prevalence of *C. trachomatis* conjunctival infection in a representative sample of the population, proven by nucleic acid amplification tests, may ultimately be required. The expense of this method of assessment could be reduced by pooling specimens [327, 328]. Diamant *et al.* have determined that if the expected population prevalence of infection is around 10%, up to fourteen specimens can be pooled per test without overly widening the confidence interval of the resulting prevalence estimate.

## 2.7 EPIDEMIOLOGY AND MECHANISMS OF TRANSMISSION

### 2.7.1 Global prevalence and geographical range

WHO defines blindness as visual acuity in the better eye of less than 3/60 with best possible refractive correction, which predicts the inability to safely walk without assistance. There are thought to be some 5.9 million people in the world who fulfil this criterion because of corneal opacity secondary to trachoma. It is the second most common cause of blindness, after cataract [25].

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<sup>xv</sup> McAdam [316], for example, suggests tossing a coin.

In addition to those already blind, an estimated ten million need surgery for trichiasis, 146 million have active infection, and some 600 million people live in trachoma-endemic areas in Africa, the Middle East, and parts of Central and South America, Asia, Australia, and the Pacific Islands [24, 25]. Because of global population growth and reductions in mortality from acute infections, Schachter and Dawson have suggested that – in the absence of effective control measures [329] – the prevalence of trachomatous blindness is likely to increase in the future [330].

### **2.7.2 Patterns of distribution**

Within endemic areas, the distribution of disease is heterogeneous. Some communities are badly affected, while others with seemingly similar community-level risk factors (see below) are not. In affected communities, clustering of active disease by sub-village [331], compound [228], and bedroom [228] has been noted. This clustering at different scales is reminiscent of fractal geometry [332].

### **2.7.3 Risk factors for active trachoma**

#### **(a) Poverty and crowding**

Taking a global view, it is readily apparent that highly-endemic trachoma is found almost exclusively in poor countries, with occasional pockets of disease in disadvantaged populations of the industrialised West. It is a disease associated with poverty<sup>xvi</sup> and sociocultural, economic or geographic upheaval [28, 333-337]. In all of these circumstances, overcrowding is typical.

At a finer epidemiological level, a number of studies have shown an association between risk of active trachoma and the number of people per unit of sleeping area, or number of people per sleeping room, in the house [338-343]. Usually, all infected members of a household harbour the same *C. trachomatis* serotype [130] and strain variant [240]. Sharing a bedroom with an active case doubles one's risk of having active disease [224]; individuals who live in endemic villages but who sleep in bedrooms by themselves are unlikely to have signs of active trachoma [228]. These findings would seem to underline the importance of intra-familial transmission, long thought to be important in sustaining trachoma [102, 344, 345]. They probably reflect an increased risk of exposure to infected secretions that results from a scarcity of living

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<sup>xvi</sup> Duke-Elder observed that trachoma was “overwhelmingly associated with the dirt, squalor and intimate proximity of the poorer classes” [28](p.1596).

and sleeping space. The “frequent, unrestricted, and indiscriminate mixing of ocular contacts or of ocular discharges” was called “ocular promiscuity” by Jones [29](p.19).

Poverty and population transition are also often associated with poor nutrition and inadequate access to water and sanitation, both of which have been examined as possible risk factors for trachoma: see below.

### **(b) Age and exposure to children**

The prevalence of both clinical active disease and laboratory evidence of infection generally decline with age after a peak in early childhood [146, 155, 331, 345-354]. In a cross-sectional survey in a hyperendemic area of central Tanzania, for example, the prevalence of active disease was 60% in pre-school children, falling to 8% in 15–35 year olds, and 2% in those over the age of 55 [331]. It has therefore been postulated that the bulk of the ocular CT reservoir in a community is found in children, and that children constitute the major source of organisms for transmission to others. Congdon *et al.* evaluated 460 adult Tanzanian women for clinical signs of trachoma, and determined whether or not they were currently taking care of children aged between one and seven years; lived with, but did not care for such children; or did not have one-to-seven year old children in the house (controls). The age-adjusted odds ratio for active trachoma rose significantly with increasing current exposure to young children [355].

As was mentioned in Section 2.4.1 (p.34), although the prevalence of active trachoma falls with age, Bailey *et al.* [155] have suggested that this may result from a decrease in the duration of each disease episode, with the difference in the incidence of disease between old and young being less marked. The intensity of inflammation, in contrast, may increase with age [155].

### **(c) Gender**

In cross-sectional surveys, females are consistently more likely to have every sign of trachoma [146, 331, 353, 356]. Usually the difference between the genders is minimal in childhood and increases with age, though one Tanzanian survey suggested that female pre-school children were already twice as likely to have constant TI as male pre-school children [357].



#### **(d) Education**

In an island-wide trachoma survey in Taiwan in 1960–61, Assaad *et al.* noted that households in which the head had a job in the professional, administrative or executive category had the lowest prevalence of active trachoma. Clerical and sales workers, workers in service and transport occupations, miners and craftsmen, and farmers and fishermen headed households with progressively higher prevalences of active disease [338]. A similar trend was noted in 1960 in Jordan [358]. Parental illiteracy has been shown to predict children having active trachoma, and cicatricial trachoma, in Ethiopia [359], and having active trachoma in the Sudan [360]. In a survey of 10,559 children under ten years of age in Mali, Schémann *et al.* found that children whose fathers had spent at least one year at primary school were significantly less likely to have active disease than those whose fathers had not. Maternal education was of marginal significance in this study [361], but the analysis may have been made difficult by the very low level of female enrolment – in 1990, less than 16% of Malian girls of primary school age were school-enrolled [362]. The association of trachoma with parental education may be confounded by exposures related to poverty (Section 2.7.3(a), p.70) or hygiene (Sections 2.7.3(e) and (f), immediately below).

#### **(e) Water and face-washing**

Trachoma is typically found in dry dusty regions where water is scarce. In a number of settings, increased distance of a household to the nearest water source has been found to correlate with an increased risk of trachoma [340, 343, 351, 363–365], and with younger age of disease [363]. The biological explanation for these associations is not proven. It is postulated that decreasing the required distance that people must travel to collect water might increase the volume of water collected and so increase water availability in the household, which might in turn increase the likelihood that some water is allocated for personal hygiene activities. Dirty faces (ocular discharge, nasal discharge, food on the face, dust on the face, flies on the face) are associated with the presence of active trachoma [343, 351, 366]<sup>xvii</sup>, and increased frequency of face washing in a household or community seems to reduce trachoma prevalence [339, 367]. Face washing might impact trachoma by decreasing the

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<sup>xvii</sup> As West *et al.* [366] acknowledge, ocular CT infection stimulates production of eye and nose discharges. Discharges attract flies. These signs could therefore be risk factors for infection, markers of existing infection, or both. The distinction may be hard to resolve, but the hypothesis that a dirty face predisposes to infection of the eye is worth supporting because facial cleanliness is potentially subject to change through education.

accessibility of infected secretions to flies and fingers, which are putative carriers of infectious inocula; by washing fly-attracting substances off both infected and yet-to-be-infected faces; or by removing chlamydia-free secretions that could provide a portal of entry for the organism into a new eye. Similarly, hand washing might reduce transmission of trachoma if fingers provide a mechanism for carriage of chlamydiae from infected to uninfected eyes [188].

Unfortunately, some of the links in this distance to water – household availability – hygiene – trachoma chain are not corroborated by published data, or are supported by some findings and not by others. For example, a recent study in Ethiopia found that trachoma was actually more common in households fifteen minutes walk or less from the nearest water source than it was in households located sixteen to thirty minutes from water [359]. The relationship between the prevalence of trachoma and daily water consumption is variable: more trachoma has been found in households with lower water use in Morocco [368] and in The Gambia [369], but no association was found in a study in Tanzania [340]. Several studies have failed to find an association even between the distance to water and daily per capita water usage [340, 368]. It may be that when water availability is restricted, families value it too much to waste it washing clothes or faces, and so do not use much even when a source is located near the house [363]. Cairncross and Feachem have suggested that there may be a plateau effect: water consumption increases as the distance to the source decreases, up to the point at which the journey time from the household to the source and back is reduced to thirty minutes. According to their hypothesis, if the distance to water is reduced still further, no additional increase in consumption is seen until the source is actually located within the compound [370].

It is possible that some other socioeconomic or environmental factor is linked to proximity to water, and independently protects against trachoma. In other words, distance to water may be a confounder. In nineteenth century London, trachoma was a problem of sufficient magnitude to inspire the foundation of Moorfield's Eye Hospital [371], yet few Londoners then or now would complain that the city suffers from a lack of rainwater. From a trachoma control point of view, the important question is this: does provision of water sources closer to the household or community, or conducting an intervention to encourage improved personal hygiene actually impact trachoma?

Unfortunately, no rigorously controlled intervention study to evaluate the effect of an improved water supply on the prevalence or severity of trachoma has been performed

[188, 363]. A community-randomised trial examining the effect of an intensive face-washing education programme on the prevalence of trachoma has shown such an intervention to be effective in reducing the prevalence of severe active trachoma in children [367], and will be discussed in Section 2.8.3 (p.84).

#### **(f) Flies and latrines**

Synanthropic flies (flies that live in close proximity to humans) have been suspected to be vectors of trachoma for more than 400 years [188]. Recently, several pieces of evidence seem to have confirmed this association.

In hand-net collection studies in trachoma-endemic villages in The Gambia, the bazaar fly (*Musca sorbens*) has been shown to make 90% of fly-eye contacts, with the house fly (*M. domestica*) accounting for the remainder [372]. These fly species are able to carry viable *C. trachomatis*, both externally and in their gut, and to transmit active trachoma from the eyes of experimentally-infected to disease-free guinea pigs [373].

In trachoma-endemic communities, household fly density, as measured by the ‘fly board’ method [374], is significantly associated with active trachoma [374], with intense active trachoma (TI) [351], and with a positive DFA for ocular CT infection [146]. The number of flies landing on an individual’s face during a five-second facial inspection is an even stronger risk factor for trachoma, as well as being easier to determine than a household fly count [375]. Emerson *et al.* found that two of 395 flies caught from the eyes of children with clinical evidence of active trachoma in The Gambia were PCR-positive for *C. trachomatis* plasmid DNA [372]. Some trachomatologists have used this unexpectedly low level of chlamydial DNA carriage by flies to downplay the suggestion that they are important in transmission. However, in areas hyperendemic for malaria, prevalences of 1% or less for *Plasmodium falciparum* infection of *Anopheles* mosquitoes are not considered unusual [376], and *Plasmodium* has a much more complex relationship with its mosquito intermediate host than the simple mechanical transfer hypothesised for carriage of *Chlamydia* by eye-seeking flies.

The most useful data on the role of flies in transmission come from intervention studies. Two community randomised trials in The Gambia have shown a reduction in the number of new prevalent cases of active trachoma in villages in which flies were controlled with insecticide [377, 378]. Because *M. sorbens* preferentially breeds on human faeces [378] but is not found emerging from the drop holes of pit latrines [372],

and because a number of surveys have associated having a household latrine with decreased risk of disease [341, 351, 359, 364], it seems intuitive to suggest that improved availability and use of latrines could reduce muscid fly density and the prevalence of trachoma. A community randomised trial designed to demonstrate this showed evidence of such an effect, but lacked sufficient power to definitively prove the impact of the intervention [378].

#### **(g) Cattle**

In Kongwa, children between the ages of one and seven years are more likely to have active disease if the family herds cows [340]. Cattle herding is also associated with a positive DFA [146]. De Sole [379] undertook a cross-sectional study in Ethiopia, examining the prevalence of active trachoma in villages with and without cattle. Active disease was over twenty-six times more prevalent amongst cattle herders than amongst camel herders. Within cattle herding communities, trachoma was less prevalent in a village that had been formed by individuals who had lost their cattle herds in a recent drought. De Sole, whilst admitting that confounding factors (ethnicity, location, behaviour) could not be excluded, postulated that cattle ownership was associated with the transmission of active trachoma because cattle manure encouraged flies. Though there is some evidence from The Gambia that human faeces is the optimal (and preferred) larval medium for *M. sorbens*, and that the flies caught from the eyes of children are from the same population as those found emerging from human faeces, females of this species can and do oviposit on the manure of cows and other domestic animals [380]. It has therefore been suggested that positioning cattle pens away from living quarters might be effective in reducing household fly density [379, 381]. No proof of this hypothesis exists.

#### **(h) Nutrition**

In Mali, night blindness and Bitot spots – the clinical features of vitamin A deficiency – seem to be associated with active trachoma, even after controlling for socio-economic status [361]. In the only study published to date that examines the effect of overall nutritional status (measured in that study by mid upper arm circumference), no association was found between malnutrition and the presence of either active or severe active trachoma [382]. However, this study had a relatively small sample size. Severely malnourished children are known to have low peripheral blood

concentrations [383] and abnormal function [384] of T-lymphocytes, as well as abnormal delayed-type hypersensitivity responses [384]: the possible link between nutrition status and trachoma may warrant re-examination.

#### **2.7.4 Extraocular reservoirs of *C. trachomatis***

From 128 children living in three trachoma-endemic villages in Egypt, Malaty *et al.* were able to isolate *C. trachomatis* in cell culture from nasopharyngeal swabs of eight children (6%), rectal swabs of nine (7%), and conjunctival swabs of thirty-six (28%). Two children were culture positive only from the nasopharynx, and five only from the rectum [220]. A study in Kongwa District, Tanzania, examined swabs taken from inside the nostrils of 232 randomly-selected children; sixty-two (27%) were positive for *C. trachomatis* DNA by PCR [246]. Nasal swabs were more likely to be positive in children with clinical evidence of active disease, or who had PCR-positive eye swabs.

No animal reservoirs of human *C. trachomatis* strains have been identified [43, 135, 385].

#### **2.7.5 Mechanisms of transmission**

The actual mechanisms of transmission of ocular *C. trachomatis* infection are unproven. Factors associated with increased risk of active disease suggest that flies, fingers, and fomites could be involved in carriage of EBs from infected to uninfected eyes within the family environment. The potential role of droplet transmission seems to have been ignored. Given that there are good data on nasopharyngeal carriage of *C. trachomatis* [220, 246], and that the aerosol route is accepted as a transmission mechanism for *Chlamydia psittaci* (*Chlamydophila psittaci*) and *C. pneumoniae* (*Chlamydophila pneumoniae*) [44, 386], this possibility probably deserves investigation.

#### **2.7.6 Risk factors for cicatricial trachoma**

##### **(a) Age**

The prevalences of TS and TT [186, 189, 339, 346, 350, 352-354, 387-389] and trachomatous corneal opacity [189, 346, 387, 388] all increase with age, as would be expected from the natural history model outlined in Section 2.2 (p.31). A 1987 survey of a village in the Nile Delta appears to show the prevalence of cicatricial disease rising more or less linearly with increasing age above the 3–5 year age group, with 90% of residents aged twenty-five or over showing moderate or severe conjunctival scarring

[350]. Munoz *et al.*, though, used age-specific prevalence data from a large cohort of Tanzanian women to estimate age-specific incidences of conjunctival scarring, trichiasis and corneal opacity in a hyperendemic area. The incidence of each condition seemed to increase with age, suggesting that the chronic manifestations of trachoma develop at an accelerating pace as individuals get older [387].

Surgical management of trichiasis will be discussed in Section 2.8.1 (p.80). In the Sultanate of Oman, trichiatics over sixty have a higher incidence of post-surgical recurrence than their younger counterparts [390].

### **(b) Gender**

Conjunctival scarring occurs more commonly in women [102, 171, 186, 331, 348, 350, 353, 354, 358, 359], as does trichiasis [331, 339, 348-350, 353, 364, 389, 391, 392]. In fact, women may have up to a four-fold increased risk of trichiasis compared to men in some areas [331]. In cohorts of patients who already have trichiasis, females have a significantly increased risk of recurrence of trichiasis after corrective lid surgery [390], and of developing visual impairment [392] compared to males. A national survey of blindness and low vision in The Gambia suggested that women were three and a half times more likely than men to have visual impairment as a result of trachoma [393].

### **(c) Poverty**

Turner *et al.* conducted a case-control study of trichiasis in rural Tanzanian women. They found a positive association between a subject having trichiasis and the number of deaths amongst her children. It seems likely that this finding relates to, for example, the socioeconomic status of the mother or the family's access to health care, rather than representing a causal association [391]. In Tunisia, Dawson *et al.* found that type of housing was associated with the presence of conjunctival scarring or trichiasis in people over fifteen years of age, with those living in single-family dwellings, multiple-family dwellings and tents having progressively higher prevalences of these signs [346].

### **(d) Education**

In Tanzania, adult education sessions are held in many villages. Topics may include nutrition, disease prevention and health, child care, and literacy. Turner *et al.*

identified never having attended these classes as a risk factor for trichiasis [391]. It is possible, though, that this association is confounded by socioeconomic status.

#### **(e) Family history and genetics**

Tuner *et al.*'s study suggested that women with trichiasis are three and a half times more likely than age-matched control women to have a mother who had trichiasis [391]. Genetic analysis was not performed, but it could be hypothesized that genetic polymorphisms at loci important in hypersensitivity or scarring might be involved. Alternatively, the predisposition of members of certain families to develop complications of trachoma could be another marker for socioeconomic status, or just demonstrate the importance of transmission of infection between family members.

#### **(f) Cumulative CT-induced conjunctival inflammation**

The excess risk of scarring and trichiasis borne by women is usually thought to result from increased exposure to ocular *C. trachomatis* infection, related to the traditional role of women as primary caretakers of young children. Congdon *et al.* evaluated the prevalence of signs of trachoma in Tanzanian women with different levels of exposure to young children (as determined at the time of the survey), and found no trend for cicatricial trachoma, though there were significant associations between caring for children and active trachoma in women (mentioned above), and between trichiasis and currently caring for at least one child with active disease [355]. Obtaining accurate information on chronic exposures (such as lifetime contact with young children) is inherently difficult, and was not attempted in this study. In fact, few papers examine environmental risk factors for the late complications of trachoma.

The few longitudinal studies that have been published seem to support a link between inflammatory trachoma and later development of scarring. In Tunisia, even the presence of moderate to severe inflammation at a single examination carried an increased risk of severe conjunctival scarring fourteen or more years later [330]. Another study in Tanzania [186] suggested that constant, severe trachoma (defined as the presence of severe inflammatory trachoma (TI) on at least three of four examinations over the course of a year, at baseline) was associated with the presence of conjunctival scarring after a seven year interval. It is unclear whether these individuals have high levels of exposure to infection, an increased susceptibility for development of severe inflammation, or both [186].

However, West *et al.* calculated the population attributable risk percent (the proportion of disease in the study population attributable to the exposure) of their constant, severe trachoma variable, and estimated it at only 28%. This implies that most scarring occurs in individuals who do not have this sign, and that control measures should not be targeted solely at those with prolonged, severe disease [186].

#### **(g) Cumulative exposure to other conjunctival irritants**

In the Turner *et al.* study, sleeping in a room with a cooking fire during the childbearing years was associated with nearly double the risk of trichiasis [391]. In Saudi Arabian men, kohl (an ancient eye cosmetic made from crushed lead sulphide ore and applied to the inside of the eyelid with a special applicator or the fingers [394]) has been identified as a risk factor for both active trachoma and any trachoma [388]. Whether eye powder or smoke induce chronic conjunctival inflammation and thereby contribute to scarring, or whether – in the case of kohl – sharing of the applicator is a transmission mechanism, is not known. However, foreign bodies resembling carbon and silica particles have been noted in the conjunctival substantia propria of South African trichiasis patients [395], suggesting at least that exogenous compounds can penetrate the conjunctival epithelium.

## **2.8 MANAGEMENT**

The only effective treatment for corneal blindness is keratoplasty, which generally involves the placement of a full thickness corneal graft. In the trachomatous eye, reduced tear secretion, corneal vascularisation and distortion of the lid all tend to worsen graft prognosis. Even though several groups have managed to achieve moderate success using corneal grafting for trachomatous corneal opacity [144, 396-398], the logistical difficulties, necessary financial resources, and disappointing long-term outcomes make establishing such a programme a low priority for most trachoma-endemic countries.

The focus of trachoma control programmes therefore needs to be preventative [397]. Unfortunately, a vaccine capable of invoking useful immunity to ocular chlamydial infection is unlikely to become available within the next decade. Current strategies for the management and control of trachoma are aimed at interrupting the pathogenic cascade that leads to blindness: reduction in the transmission of infection from person to person through fly control and the removal of infected ocular secretions



from children's faces, treatment of established infections with antibiotics, and correction of trichiasis and entropion before patients become blind. Because the latter group are at most immediate risk of developing trachomatous visual impairment, surgery is the intervention that should be undertaken most urgently in a trachoma-endemic area. The acronym 'SAFE', used to encapsulate the trachoma control strategy recommended by the World Health Organisation, places the four elements of control in order of their priority: Surgery for trichiasis and entropion, Antibiotics for active disease, and Face washing and Environmental improvement to reduce transmission.

### **2.8.1 Surgery**

Surgical treatment of trichiasis and entropion aims to stop abrasion of the cornea by in-turned lashes, and thereby minimise ongoing deterioration in vision. Many techniques have been described, ranging from the minimally invasive, such as the use of sticking plasters to adhere offending lashes to the skin of the lid and orbital margin [399], through to the frankly disfiguring, such as the complete excision of the ciliary margin of the lid with granulation of the raw edge advocated by Stellwag in 1883 [400]. Other procedures nominated include part-resection of orbicularis oculi with placement of sutures to evert the lid margin [400]; splitting of the lid in the frontal plane with interposition of a pedicle of skin, muscle or mucous membrane between the two layers [400, 401]; destruction of lash follicles by cryotherapy [402-407]; electro-epilation [390, 406, 407]; X-ray epilation [408]; wholesale removal of the tarsal plate and orbital septum [409]; tarsal grooving (the Streatfield-Snellen procedure) [410]; eversion splinting (grey line split) [410, 411]; tarsal advance (lid split) [410]; tarsal advance and rotation (Collin's modification of the Trabut procedure) [407, 410, 412, 413]; tarsal advance and graft [407]; posterior lamellar tarsal rotation [414, 415]; and bilamellar tarsal rotation (upper lid Wies or Ballen operation) [407, 410, 416, 417]. Reacher undertook two randomised clinical trials [407, 410]. He concluded that tarsal plate rotation (in which a transverse incision is made through the tarsus a few millimetres from the lid margin, and sutured under moderate tension so as to evert the lashes) was the superior technique, with an estimated 77% success at twenty-one months after surgery [407]. Comparable results seem to be obtained regardless of whether the incision is full thickness (bilamellar tarsal rotation) or spares the skin of the lid (posterior lamellar tarsal rotation) [414, 415]. Tarsal plate rotation is a simple, rapid procedure which can be performed effectively by paramedical staff. This is fortunate,

since in Africa, for example, there is only one ophthalmologist for every million people [418].

Traditional treatment for trichiasis often consists of mechanical epilation of ingrowing lashes using home-made forceps. Some control programmes recommend this to patients with minor trichiasis. Bowman *et al.*, however, suggest that surgery might be a better strategy, particularly because of the difficulties involved in monitoring disease progression in patients living in remote communities [419].

Achieving high uptake of surgical services often proves difficult. Programmes in Malawi [420], Tanzania [421, 422], Egypt [392] and The Gambia [189] have all reported that less than 50% of those offered correction of trichiasis actually present for surgery, even when transport to the health facility is provided by the programme and operations are performed free of charge. Uptake of surgery is lower for women than men [392]. Village-based surgery, which has been shown to produce good operative outcomes [414], may improve uptake by saving time for the patient and reducing fear of the procedure [415].

### **2.8.2 Antibiotics**

Before the introduction of sulphonamides in 1938, infection with the trachoma agent was thought to be incurable. Unfortunately for patients, this did not prevent ophthalmologists attempting to inactivate disease by scrubbing the upper fornix and palpebral conjunctiva with pointed crystals of copper sulphate fixed to the end of wooden probes. This would be repeated “daily, month after month, year after year, until the disease (became) quiet” [28](p.1620). This treatment, Duke-Elder remarked, was “by no means painless” [28](p.1620).

Fortunately, the arrival of antibiotics heralded a new approach. The opportunity was not ignored. In fact, as Dawson *et al.* noted, “since the advent of the sulphonamides, each antimicrobial drug which became available has found some endorsement for the treatment of clinical trachoma” [423](p.581). In 1950, Freyche reviewed available evidence supporting the use of tyrothricin, penicillin, streptomycin, bacitracin, aureomycin, and chloromycetin. He included 194 references, and alluded to a further “number of papers so great that even their mere enumeration would be tedious” [424](p.523). Since then, the volume of published work, and the number of different antiseptics and antibiotics suggested or tested for the management of trachoma have both continued to grow. The list now includes boric acid [425-429], rifampicin

[426-428, 430-433], erythromycin [425, 428, 429], spiramycin [432], chlortetracycline [425, 433-437], oxytetracycline [432, 434, 438], oxytetracycline/polymixin [245, 439], tetracycline [182, 247, 426, 427, 429, 434, 435, 437, 440-444], and meclocycline [445], all used topically; oral preparations of rifampin [446], sulfonamides [185, 423, 424, 436, 440, 441, 444, 447-450], tetracycline [441, 451], doxycycline [444], minocycline [452, 453], amoxicillin [169], erythromycin [169, 442, 448] and azithromycin; and intramuscular benzathine penicillin [454] and sulphamethoxypyridazine [454, 455]. New compounds are still being trialed. Recently, studies in Japan have suggested that tranilast, from the leaves of *Nandia domestica* (sacred bamboo), and FNQ3, a synthetic analogue, may have *in vitro* efficacy against chlamydiae [456].

Most of these compounds have never been formally tested (by today's standards) against active trachoma. Beginning in the late 1930s, though, mass distribution of sulphonamides was used for trachoma control in American Indian communities. In the 1960s, these drugs were evaluated against placebo for the treatment of active trachoma, with mixed results [423]. Though given part of the credit for the dramatic fall in prevalence seen in indigenous Americans during the twentieth century [457], sulphonamides later fell from favour because of the high incidence of adverse reactions (including erythema multiforme, Stevens Johnson syndrome and renal complications) associated with their use [334, 446], and are now no longer recommended for active trachoma. Topical tetracycline and azithromycin are both in current use, and will be discussed in this review. Erythromycin and amoxicillin are given for the treatment of TI in some control programmes that do not have access to azithromycin, but neither has been subjected to a randomised controlled trial. Why, though, are antibiotics used at all?

In the context of trachoma, antibiotics are used with two overlapping goals. Firstly, agents that are effective against *C. trachomatis* are prescribed to individuals with clinical signs of active disease. The objectives here are to lessen the risk of the patient's close contacts becoming infected, relieve symptoms, and shorten the period of conjunctival inflammation [132]. The last of these would have the theoretical advantage of decreasing the stimulus for the deposition of conjunctival scar. There are no published data showing that the administration of any antibiotic achieves any of these things.

Secondly, antibiotics are distributed to subsets of or whole populations, to try and reduce the community load of *C. trachomatis*, and therefore achieve sustained

reductions in the prevalence of disease. The ultimate goal is to reduce visual loss due to trachoma [132, 211]. There is some recent evidence to suggest that mass azithromycin distribution can lower the community burden of *C. trachomatis* for at least twelve months [169], as will be discussed later, but none so far proving that antibiotics can reduce the incidence of trachoma blindness.

Tetracyclines are active against *Chlamydia trachomatis*. In the 1950s, mass treatment campaigns using tetracycline eye ointment were used for trachoma control in a number of countries [458]. As part of WHO-supported monitoring of the Moroccan programme in that decade, a large number of schoolchildren were followed to assess outcome after being given ointment twice- or three-times daily for sixty days. Nearly 80% clinical cure was reported [459]. Results elsewhere were less encouraging [435, 440].

No placebo-controlled trials of topical tetracycline were undertaken until a 1965–67 study by Dawson *et al.* [180]. This trial was conducted in American Indian children resident at a boarding school. Topical tetracycline was applied three times daily for six weeks, and produced a greater reduction in the prevalence of clinical activity than a topical placebo, both at the conclusion of therapy and one month later. However, the prevalence of active disease continued to fall in intervention and placebo groups alike, and there was no significant difference in outcome five months after treatment finished: this was attributed to the favourable hygienic environment of the school [180]. A group-randomised trial in Taiwanese schoolchildren concluded that evaluating the advantage of tetracycline over placebo was made difficult by the high incidence of “spontaneous cure among the controls, coupled with a high rate of reversion from healed to active trachoma among the treated subgroups” [435](p.569). A non-randomised trial in Tunisia appeared to show that tetracycline was superior to boric acid (a mild antiseptic, used in that study as a control) in the resolution of both clinical activity and infection [427]; and a randomised controlled trial by Attiah *et al.* showed a statistically insignificant advantage of tetracycline over no treatment [438]. On the whole, data do not prove that tetracyclines lower the prevalence of disease or infection, but merely suggest that some benefit might be derived from their use [460]. Two reasons for this weak effect can be proposed.

First, from the recipients’ perspective, ocular tetracycline is not an ideal therapy. The ointment is irritant to the conjunctiva, difficult and often messy to self-administer, and for refractive reasons blurs vision for a few minutes after application. Prolonged

administration is recommended. It is likely that the majority of individuals prescribed six weeks continuous or six months intermittent treatment do not complete the course, particularly if they are initially asymptomatic. Most individuals targeted for trachoma chemotherapy do not feel ill [461]. Second, the presence of *C. trachomatis* at extraocular sites such as the nasopharynx and rectum has been demonstrated in several surveys of trachoma-endemic communities [182, 220, 246]. Instillation of antibiotics into the conjunctival sac alone is unlikely to clear infection from these areas [446, 462]. Rapid autoinfection from extraocular reservoirs after successful topical treatment then becomes a theoretical possibility. Whether extraocular chlamydial reservoirs are truly significant for the epidemiology of trachoma is still unclear.

If topical treatment works, twice daily application may be just as effective as thrice daily [459], and an intermittent schedule (where ointment is applied, for example, twice a day for three consecutive days, repeated every four weeks over twenty weeks) may be more effective in the long term than continuous treatment five days a week for twelve weeks [459]. Intermittent treatment has the additional advantage of requiring less ointment [459]. Before the optimal duration and frequency of topical tetracycline administration could be definitively established, it was realised that attempts to prevent trachoma blindness using tetracycline would be resource intensive, require prolonged effort, and might have disappointing results [24, 438]. Community-based tetracycline distribution programmes were abandoned [24].

The demonstration that a single dose of oral azithromycin is at least as effective as prolonged courses of topical tetracycline has dramatically altered the approach to trachoma chemotherapy in a number of endemic countries. Presently, eight countries are fortunate to receive donations of azithromycin for trachoma control from the International Trachoma Initiative (ITI); others, like Australia, can afford to provide it under the national health budget. Elsewhere, despite its imperfections, topical tetracycline remains the first line treatment for active trachoma, for want of a more attractive alternative. Azithromycin is discussed under its own heading in Section 2.9 (p.86).

### **2.8.3 Face washing**

Because dirty faces are associated with active trachoma, West *et al.* undertook a community-randomised intervention trial to examine the impact of promoting face washing as an adjunct to mass treatment with tetracycline eye ointment. Three pairs of

hyperendemic villages matched on maternal education and baseline prevalences of clean faces and clinical trachoma in young children were enrolled. Every resident of both intervention and control villages was offered topical tetracycline once daily for thirty days. In intervention villages, an intensive one-month participatory campaign was conducted to encourage washing of children's faces [367]. The campaign included a seminar with village leaders; a series of neighbourhood meetings to build consensus on the importance of face washing, develop plans, and – subsequently – to review progress; a school programme to raise awareness of facial cleanliness; seminars with traditional healers; and presentations (by schoolchildren and the local traditional dance group) of songs, dramas and drawings related to trachoma and face washing [367, 463]. Antibiotics reduced the prevalence of active trachoma in children in both the intervention and control groups. The proportion of children with sustained clean faces at follow-up was higher in the intervention group (35%) than the control group (26%), and in two of three intervention villages, the return in the prevalence of severe trachoma (defined by West *et al.* as fifteen or more follicles or the presence of inflammation that obscured all the deep tarsal vessels, in the examined eye) towards the baseline level was slowed. The face washing promotion campaign had no effect on the prevalence of TF [367]. In the majority of trachoma control programmes, resources to conduct a campaign of similar intensity are unavailable. The impact of less energetic face washing education efforts is not known.

#### **2.8.4 Environmental improvement**

There is general agreement in the literature that improvements in a community's standard of living are associated with a decrease in trachoma transmission [346, 364, 462, 464-466]. Socioeconomic development and increased living standards are believed to be responsible for the disappearance of trachoma from Europe and North America [211].

Unfortunately, merely waiting for socioeconomic development to take place in trachoma-endemic areas will not help to prevent trachoma blindness. Some work has been done to try and identify the actual components of 'development' that reduce the prevalence of trachoma. Fly control appears promising, but studies have so far failed to demonstrate effectiveness for an intervention that might prove sustainable. Further work is required. Improvement in water supply, which is a recommended part of the SAFE strategy and popular with communities, is yet to be subjected to controlled trials.

## 2.9 AZITHROMYCIN

Azithromycin is an azalide macrolide. It is a derivative of erythromycin, differing only through the addition of a methyl-substituted nitrogen at position 9a of the aglycone ring. This expands the ring to fifteen members, increases acid stability (and so increases absorption), enhances tissue penetration, and broadens the antibiotic spectrum, compared to the parent compound. It is effective against *C. trachomatis* both *in vitro* [467, 468] and *in vivo* [469].

### 2.9.1 Spectrum of activity

In addition to its activity against *Chlamydia*, azithromycin has some activity *in vitro* against *Staphylococcus aureus*, *Streptococcus pneumoniae*, *S. pyogenes*, and other *Streptococcus* spp., *Haemophilus influenzae*, *H. parainfluenzae*, and *H. ducreyi*, *Campylobacter* spp., *Branhamella cararrhalis*, *Escherichia coli* and other members of the Enterobacteriaceae, *Bacteroides fragilis*, *Fusobacterium* spp., *Neisseria gonorrhoeae*, *Bordetella pertussis* and *B. parapertussis*, *Mycoplasma pneumoniae* and *M. hominis*, *Ureaplasma urealyticum*, *Leigonnella pneumophila*, *Borrelia burgdorferi*, *Mycobacterium avium-intracellulare*, *Toxoplasma gondii*, *Treponema pallidum*, *Cryptosporidium*, and *Plasmodium* spp. [470-474].

### 2.9.2 Mechanism of action

Azithromycin binds to the 50S ribosomal subunit of susceptible organisms, and thereby interferes with bacterial protein synthesis.

### 2.9.3 Pharmacokinetics

Azithromycin is absorbed rapidly from the gastrointestinal tract [475] and distributes widely throughout the body, except to the CSF. Though initial studies suggested that food in the stomach could dramatically decrease bioavailability [476], later clinical trials with the commercial formulations of azithromycin determined that it could be given without regard for meals [477]. It has an unusually large volume of distribution (31L/kg) because of its high level of tissue binding: tissue concentrations are ten to one hundred times higher than those of serum [478]. Azithromycin is concentrated intracellularly by a number of cell types [479, 480]. This may help to explain the drug's extraordinary efficacy against the (intracellular) chlamydiae. Tissue depletion half life has been estimated at 2–4 days [478].

Two studies in Saudi Arabia provided information about particular aspects of azithromycin's pharmacokinetics relevant to its use in trachoma control. Tabbara *et al.* obtained the assistance of sixty patients (mean age sixty-six years) undergoing cataract surgery, each of whom was given a single 1g oral dose of azithromycin at a variable time prior to surgery. Tear samples and conjunctival biopsy material were obtained at operation. All tears collected four days or less after azithromycin, and all conjunctival specimens collected fourteen days or less after azithromycin contained levels of azithromycin above the MIC<sub>90</sub><sup>xviii</sup> for *C. trachomatis* [482]. Karcioğlu *et al.* studied fourteen school-age Saudi children with active trachoma. They gave each child a single dose of 20mg azithromycin per kilogram bodyweight, and repeatedly sampled subjects' tears and plasma over the following six days. The concentration of azithromycin in tears was within or above the MIC<sub>50</sub> range for *C. trachomatis* (0.03–0.25µg/mL) at six days for all 12 patients for whom data were available<sup>xix</sup> [483].

#### 2.9.4 Indications

The prolonged half life and intracellular concentration of azithromycin make it an extremely useful therapeutic tool. A single oral dose of 1g azithromycin is as effective as seven days of twice-daily oral doxycycline in the treatment of lower genital tract *C. trachomatis* infections [484-486]. Such a dose clears urethral and cervical chlamydial infection in almost 100% of cases [469]. Since it is also effective against *Neisseria gonorrhoeae*, single dose azithromycin is now employed in some countries for the syndromic treatment of sexually transmitted infections. In the West, the drug is also used for infections of the respiratory tract, middle ear, skin and soft tissue.

In the developing world, the expense of azithromycin tends to exclude it from routine use in the management of any of these conditions – a one gram dose costs over £7 in the UK. Only the existence of a donation programme makes azithromycin a viable option for trachoma control.

#### 2.9.5 Formulation

ITI currently provides Pfizer-donated azithromycin (Zithromax™, Pfizer, New York, New York) to trachoma control programmes in Egypt, Ethiopia, Ghana, Nepal,

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<sup>xviii</sup> The minimal inhibitory concentration-50 (MIC<sub>50</sub>) is the concentration of antibiotic which produces a 50% reduction in the inclusion count compared to a drug-free control culture; the minimal inhibitory concentration-90 (MIC<sub>90</sub>) is the concentration which produces a 90% reduction [481].

<sup>xix</sup> Two patients failed to complete the study.



Niger, Mali, Morocco, Sudan, Tanzania, and Vietnam. Zithromax for donation is manufactured as 250mg tablets and bottles of cherry- or banana-flavoured powder for suspension (paediatric oral suspension, POS). When reconstituted according to directions, the concentration of the suspension is 40mg/mL.

### **2.9.6 Efficacy against trachoma**

Three trials established the efficacy of azithromycin in the treatment of active trachoma.

Bailey *et al.* [182] studied 194 non-pregnant, non-lactating individuals with active disease (aged nine months to sixty years) in The Gambia. Patients were randomised by room to a single dose of azithromycin 20mg/kg or 1% ocular tetracycline ointment applied twice daily under supervision for six weeks. Those with severe inflammation (TI) also received oral erythromycin four times a day for four weeks, in line with Gambian national guidelines. More than 98% of scheduled treatments were witnessed. No serious adverse reactions were observed. At six months after the commencement of treatment, active disease was absent in 78% of the azithromycin group and 72% of the tetracycline group. This difference between the two treatment groups was not statistically significant.

Tabbara *et al.* [247] conducted a randomised controlled trial comparing azithromycin and topical tetracycline in sixty-four Saudi children aged between six and fourteen years, all of whom had active trachoma at baseline. Azithromycin was given as a single oral dose of 20mg/kg; tetracycline was administered by a school teacher twice daily, five days weekly, for six weeks. Conjunctival scrapings were collected for Giemsa staining and DFA at baseline and twenty-four weeks, and masked follow-up examinations were undertaken at four, eight, twelve and twenty-four weeks. There were no significant differences, either clinically or microbiologically, between the treatment groups.

In a study of 168 children with active disease in northern Egypt, Dawson *et al.* [245] compared four different treatment strategies. Blocks of eight children were randomised to (1) application of 1% oxytetracycline / 10,000 units/g polymixin ointment once daily for five consecutive days in every twenty-eight days for six cycles; (2) one dose of azithromycin 20mg/kg (3) azithromycin 20mg/kg once-weekly for three consecutive weeks; or (4) azithromycin 20mg/kg once in every twenty-eight days for six cycles. There were no significant differences in clinical or microbiological cure

rates between the various groups at any of four follow-up examinations following the commencement of treatment.

A fourth trial, conducted in a trachoma-endemic area in Turkey by Guzey *et al.*, selected ninety-six subjects with active trachoma for treatment with either a three-day course of 10mg/kg azithromycin suspension per day, or eight weeks twice daily 0.5% oxytetracycline/polymyxin eye ointment. Unfortunately, criteria for diagnosis with active trachoma were not well defined in this paper [439].

In all of these trials, sample sizes were almost certainly too small to be able to demonstrate statistically significant differences in the effect of treatment [182]. However, the trials suggest that, under supervised conditions ensuring near-maximal compliance with prolonged topical treatment courses, single dose oral azithromycin is at least as effective as topical tetracycline.

When treatment is restricted to individuals with clinical signs, it is probable that not all ocular chlamydial infections are treated, and re-emergence of disease in the community is likely to be rapid. The 'Azithromycin in Control of Trachoma' (ACT) trial was therefore undertaken to examine the impact of mass treatment with azithromycin [169]. The study was a community-randomised trial in trachoma-endemic areas of The Gambia, Egypt and Tanzania, using pairs of villages matched on the prevalence of active trachoma in children aged one to ten years. In villages randomised to tetracycline, supervised once daily topical tetracycline was offered to each village resident for six weeks. In villages randomised to azithromycin, 20mg/kg of that drug was offered to each resident once weekly for three weeks: three doses were offered to ensure maximum coverage. Women of childbearing age were given oral erythromycin or amoxicillin for fourteen days instead of azithromycin. In each country, village-wide LCR positivity rates at one year were substantially lower than at baseline with both treatments. The reduction in laboratory positivity was greater with azithromycin than with tetracycline, though the difference was not significant. Additionally, in each country and in each disease category, the proportion clinically free of active disease twelve months after treatment with azithromycin was at least equal to the proportion free of active disease after treatment with tetracycline [169]. Data from the Egyptian arm suggest that the effect of either azithromycin or tetracycline on infection was far greater than the effect on disease at that site [327].

### **2.9.7 Is azithromycin the antibiotic of choice?**

Is azithromycin actually more effective than tetracycline? Should it be seen as the antibiotic of choice? For three reasons, the answer to both these questions is: probably yes.

First, in studies indicating equivalent efficacy of azithromycin and topical tetracycline, high tetracycline compliance rates were achieved through careful supervision of antibiotic administration. Mass daily or twice daily directly-observed application of tetracycline eye ointment is not achievable outside the research setting. In trachoma control programmes, one or two tubes of tetracycline are generally given to the individual to be treated, or their parent, with a brief demonstration of the method of application. For reasons that were outlined above, it is likely that many do not complete a full six week course. In contrast, azithromycin is simple to take, simple to administer, and single dose treatment can be directly observed even in large-scale distribution programmes. Bowman *et al.* [487] undertook a randomised controlled trial comparing one directly observed dose of 20mg/kg azithromycin against an unsupervised six week course of twice daily ocular tetracycline, in the treatment of children with active disease. Children allocated to azithromycin were significantly more likely to be clinically cured at both ten weeks and six months post randomisation.

Second, because azithromycin is administered systemically, it is likely that it eliminates infection from both eyes and from extraocular sites [211], and thereby reduces the likelihood of ocular reinfection by autoinoculation. Topical tetracycline is unlikely to have a similar effect.

Third, azithromycin is active against other bacterial infections, most notably those of the genitourinary tract. It also has antiplasmodial action: a significant reduction in the prevalence of parasitaemia, febrile parasitaemia, and splenomegaly has been reported in a Gambian community receiving triple dose azithromycin for trachoma [488]. Goodwill generated by the incidental treatment of such conditions can be of great benefit in developing community support for trachoma control [489], and should not be underestimated when considering the value of azithromycin to the SAFE strategy.

### **2.9.8 Adverse effects**

Treadway and Pontani collated data from forty-three open randomised controlled trials conducted on four continents, comparing the incidence of adverse events in 2,655 children treated with azithromycin 10mg/kg once daily for three days, with that in 1,844

children treated with one of nine comparator antibiotics [490]. All children were between the ages of six months and sixteen years, and all were undergoing treatment for suspected or confirmed bacterial infections. Adverse events occurred in 232 (8.7%) of the children treated with azithromycin, and 180 (9.8%) of those treated with comparator. Most adverse events were mild or only moderately severe, and most were gastrointestinal. Similarly, phase II and III trial data from fourteen countries presented by Hopkins [491] showed a 12.0% incidence of side effects amongst 3,995 patients aged from two to ninety-four years treated with single or multiple dose azithromycin: significantly lower than that associated with comparators.

In the Gambian arm of the ACT study [169], compliance was over 98% for azithromycin, while nearly 95% of the villagers randomised to tetracycline received twenty-eight or more applications. Morbidity surveys of all subjects aged three months to fourteen years were conducted before treatment commenced, and at weekly intervals for four weeks thereafter. During follow-up, the azithromycin group had 20% fewer overall illnesses, fever and headache episodes and 40% fewer diarrhoea and vomiting episodes than did the tetracycline group [492]. The trial was conducted during a high malaria transmission season; the fact that fewer episodes of systemic illness were reported in the azithromycin treated group than in the group treated with eye ointment was thought to be due to the antimalarial effect of azithromycin [492].

Mass treatment of all children aged one to ten years in far-western Nepal has been associated with a similar overall beneficial effect, despite a low prevalence of malaria. In children who had not previously been given azithromycin, the proportion whose parents reported impetigo, diarrhoea, or abdominal pain was lower ten days after treatment than at baseline; the proportion of control group children whose parents reported these symptoms did not change [493]. Other trials of azithromycin against trachoma also suggest that it is well tolerated in trachoma-endemic communities [182, 247, 439].

### **2.9.9 Issues in the use of azithromycin**

#### **(a) Who should it be given to?**

Determining who to treat is a priority for research [494]. The poor correlation between clinical trachoma and chlamydial infection, and uncertainty about the relative importance of different age and gender groups in transmission, make it difficult to decide who needs azithromycin and who does not. Various options have been

proposed, including blanket treatment of all residents of endemic areas [169], mass treatment of one to ten year-old children [495, 496], targeted treatment of children aged from one to ten who have clinical evidence of active trachoma, together with treatment of other members of their households [495, 496], and treatment of children with trachoma and their household contacts who are children [497, 498]. Only the study by Holm *et al.* in Nepal has provided empirical data on the relative effectiveness of different treatment strategies. That study, which compared treatment of all children against targeted treatment of children with clinically active disease plus their households, found no significant difference in the effect of the two strategies on the prevalence of either active disease or *C. trachomatis* infection [495].

In previous mass treatment campaigns using topical tetracycline, the formal recommendation that all children in endemic areas be treated translated to treatment only of schoolchildren in practice, since such children are easily accessible, while non-school attenders are not [429]. Unfortunately, the peak prevalence of active disease is in children below school age. Additionally, schoolchildren are likely to be healthier and to come from more wealthy families, and may therefore be less likely to have trachoma than children of the same age who are not at school. It became apparent that treatment of schoolchildren alone was unlikely to produce a useful impact on the prevalence of trachoma [435].

There has been a reluctance to consider the use of azithromycin in pregnant women, and in children below six months of age. However, a growing body of evidence suggests that the drug does not harm the developing foetus. Gray *et al.* conducted a community randomised trial of presumptive treatment for sexually transmitted diseases in pregnancy [499], with the principal object of evaluating the effect of such an intervention on maternal HIV acquisition and perinatal HIV transmission [500]. Mothers in the intervention arm, who were at varying stages of gestation, received a cocktail of antibiotics including a single dose of 1g azithromycin. Early neonatal mortality and the incidence of low birth weight were both significantly lower than in the control arm [499], suggesting that the antibiotics given actually improved pregnancy outcome. For pregnant women themselves, single dose azithromycin may produce a lower incidence of severe side-effects than a seven-day course of erythromycin [501]. Azithromycin has been recommended since January 1998 by the Centres for Disease Control and Prevention as an alternative regimen for treatment of genital chlamydial infections in pregnancy [502]. Extensive studies in animals have failed to show any

evidence of teratogenicity [503]. Based on these data, the ITI's Trachoma Expert Committee has recently recommended that the prohibition on azithromycin for pregnant women be lifted.

There are relatively few data on the use of azithromycin in children below six months of age [503]. In a small study evaluating the drug for use in neonatal chlamydial conjunctivitis, thirteen infants between six and eighteen days old received one to three once daily doses of 20mg/kg azithromycin. No adverse effects of any kind were observed [504].

#### **(b) Who should give it?**

The problem of ophthalmic understaffing in Africa has already been mentioned. In trachoma-endemic areas, few ophthalmologists, medical officers or nurses have sufficient time to devote themselves to a prevention campaign addressing just one of the many health problems for which they have responsibility. Community health volunteers have previously been involved in trachoma control activities [102, 505]. Some of these volunteers are currently contributing to the global Guinea Worm eradication campaign. As Guinea Worm disappears, the experience and motivation of volunteers should be harnessed for other health interventions [506]. A pilot study in Ghana recruited six Guinea Worm volunteers for training in the diagnosis of active trachoma and its treatment with oral azithromycin [507], with generally positive results. Attempts to involve these individuals on a larger scale might be worthwhile. True community-directed distribution, as has been employed in ivermectin distribution for onchocerciasis [508-511], may be even better.

#### **(c) How should it be given?**

Community volunteers recruited to distribute azithromycin are likely to travel by foot or by bicycle. Weighing scales are typically heavy and bulky. In Ghana, Guinea Worm volunteers were unfamiliar with the operation of analogue weighing scales, and required several days' training before they were able to accurately determine subjects' body weight [507]. Furthermore, scales are relatively expensive, prone to breakage, frequently lose calibration or perform unreliably when used on dirty uneven surfaces, and are useful in commerce, so are prone to disappear [507, 512].

Mid upper arm circumference (MUAC) [513, 514], various products of height and MUAC [513], and physical appearance (estimation of dose without taking any

measurements) [514, 515] have all been explored as alternative means for determining drug doses, but height seems to be the best proxy for weight in rural African populations. Distribution of anti-infectives by height has been successful for the treatment of onchocerciasis with ivermectin [515] and for the treatment of schistosomiasis with praziquantel [513]. A height scale can be quickly and cheaply constructed using any straight length of wood or plastic. Laminated paper tapes (based on those used in paediatric trauma centres in North America) have been trialed for general paediatric medicine drug dosing in Malawi, and could provide a lightweight and compact alternative [512].

#### **(d) Will resistance emerge?**

Broad-based distribution of azithromycin in trachoma-endemic communities seems to provide ideal conditions for the selection of azithromycin-resistant strains in a range of bacteria, since human population exposure is extensive, one-dose treatment is employed, the drug has a broad spectrum of activity, and pathogen carriage rates are likely to be high. Emergence of azithromycin resistance in ocular strains of *C. trachomatis* would reduce the effectiveness of this antibiotic in trachoma control programmes. Emergence of azithromycin resistance in other human pathogens might make the manufacturer revise its commitment to the whole donation programme.

*In vitro* studies highlight the ease with which chlamydiae can develop resistance to some antimicrobials. In 1973, rapid selection of rifampin-resistant mutants of *C. trachomatis* was noted when successive isolates were grown in increasing concentrations of that antibiotic [516]. Four passages of the L2 reference strain through McCoy cell culture containing subinhibitory concentrations of ofloxacin and sparflaxacin is sufficient for the selection of mutants highly resistant to fluoroquinolones [517]. The discovery by Lenart *et al.* of inclusion vacuoles containing both *C. suis* and *C. trachomatis* L2 (following sequential infections of the same tissue culture) raises the possibility of inter-species interaction, and – potentially – exchange of resistance genes through plasmids or chlamydial phages [518].

It is somewhat reassuring that despite decades of tetracycline use in trachoma control, tetracycline-resistant ocular CT has not yet been identified [519]. However, Mourad *et al.* reported partial erythromycin resistance in clinical isolates as early as 1980 [481], and tetracycline-resistant endocervical and urethral isolates were first reported by Jones *et al.* in 1990 [520]. In the latter study, resistance was responsible for

treatment failure in at least one of the five patients from whom the isolates were obtained, and tetracycline-resistant strains were also found to be resistant to doxycycline, erythromycin, sulfamethoxazole, and clindamycin. Most recently, urogenital *C. trachomatis* isolates resistant to doxycycline, azithromycin and ofloxacin causing clinical treatment failure have been reported [521].

Extensive use of azithromycin may also lead to the emergence of resistant strains of other organisms. Chern *et al.* studied the effect of azithromycin on the conjunctival bacterial flora in Nepalese children. Bacterial cultures were taken from the conjunctivae of 121 children at baseline and two weeks later. Subjects were randomised to receive a single dose of azithromycin 20mg/kg either after the baseline or after the follow-up swabbing. At baseline, all *S. pneumoniae* isolates recovered from both groups were sensitive to azithromycin. The proportion of children from whom pathogenic bacteria were isolated fell significantly in the baseline azithromycin-treated group, and three of seven strains recovered from this group at follow-up were resistant to azithromycin. Two of these resistant isolates were from children who had grown azithromycin-sensitive *S. pneumoniae* strains prior to antibiotic treatment [522].

In The Gambia, the impact of three once weekly doses of azithromycin 20mg/kg on carriage of oropharyngeal pathogens in children has been compared to that produced by daily treatment with tetracycline eye ointment. Oropharyngeal swabs were taken before treatment and twenty-eight days later. There was a significant reduction in carriage of *S. pneumoniae* in the azithromycin group, but no effect on the carriage of *H. influenzae*; antibiotic susceptibilities were not reported [523]. In German children with respiratory tract infections, azithromycin seems more likely than other macrolides to select for resistant strains of oral microflora [524].

Two published studies have examined the effect of azithromycin used for trachoma control on the prevalence of antibiotic resistance in non-ocular pneumococci. Working with Aboriginal children in the Northern Territory of Australia, Leach *et al.* examined the effect of azithromycin on nasopharyngeal carriage and antibiotic resistance of *Streptococcus pneumoniae* [497]. A total of 130 children with trachoma and their household contacts who were children were given a single dose of azithromycin 20mg/kg. Seventy nasopharyngeal swabs were taken from cases before treatment, and up to thirty-nine were taken at two weeks, two months and six months after azithromycin. Carriage rates were 68%, 29%, 78% and 87% at baseline and these three follow-up points respectively. The prevalence of azithromycin resistance in strains



from carriage positive children was higher at two weeks than at baseline, and higher again at two months, then fell between two months and six months [497]. However, the formulation of azithromycin used was unusual<sup>xx</sup>, follow-up was limited by the terms of the ethical approval obtained, and there was no control group: the observed change could have been produced by a number of confounding factors, such as the use of other antibiotics by cohort individuals [525].

Fry *et al.* examined antimicrobial resistance profiles of nasopharyngeal pneumococcal isolates before and after a mass azithromycin distribution campaign in far-western Nepal [493]. A total of 458 children were followed, divided amongst three groups: the ‘current treatment’ group, living in villages in which azithromycin 20mg/kg was given to each one to ten year-old child shortly after baseline swabbing, and in which azithromycin had not previously been offered; the ‘one previous exposure’ group, who had received azithromycin six months prior to baseline; and the ‘two previous exposures’ group, who had received azithromycin on two separate occasions, six months and eighteen months prior to baseline. Neither the one nor two previous exposures groups were given azithromycin between baseline and the follow-up swabs. No azithromycin resistant isolates were obtained from the current treatment or one previous exposure groups, either before treatment, or ten or 180 days afterwards. In the two previous exposures group, two of ninety-two (2%) of isolates were azithromycin resistant at baseline, and four (5%) of eighty-eight isolates were resistant at 180 days post baseline; this change was not significant [493]. This may indicate that, when the pre-existing prevalence of macrolide resistance is low, multiple courses of azithromycin are required to produce resistance in pneumococci [493].

Though it is true that azithromycin is not commonly used against *S. pneumoniae* in most trachoma-endemic countries [519], microorganisms do not respect international boundaries. Both additional specific studies and ongoing surveillance are warranted.

#### **(e) How often should it be given?**

Using a mathematical model, Lietman *et al.* estimated the required frequency of azithromycin dosing to be every six to twelve months in hyperendemic areas<sup>xxi</sup> and every twelve to twenty-four months in moderately trachoma-endemic areas<sup>xxii</sup>. Calculations, though, were based on data for prevalence of disease rather than infection:

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<sup>xx</sup> a suspension of crushed azithromycin tablets

<sup>xxi</sup> defined by Lietman *et al.* as a prevalence of active disease in children at baseline of greater than 50%

given the poor correlation between the two, analysis with the latter might generate more useful output. In addition, the model assumed universal treatment coverage, 95% treatment efficacy, structured mixing patterns of residents, homogeneous distribution of cases, and no inward migration [526]. There remains a need for empirical data on rates and routes of reinfection following azithromycin treatment for trachoma control.

## 2.10 SUMMARY

Repeated ocular infection with *Chlamydia trachomatis* is an important cause of blindness. Some details of the biology of the organism, the pathogenesis of disease and the mechanisms of human immunity have proven difficult to unravel, but sufficient information is available to permit a rational approach to diagnosis. Because clinical signs correlate poorly with the presence or absence of ocular CT, research into the epidemiology of trachoma and the effect of control interventions should be based on tests of infection status. A number of kinds of laboratory assay are available. The sensitivities of the nucleic acid amplification tests are probably at least as high as those of other assays, and their specificities are probably close to 100%. Additionally, the ability of kinetic quantitative PCR to rapidly and accurately quantify the number of copies of a DNA sequence present in a sample makes it an ideal research tool for investigating the epidemiology of this disease.

The strategy used for the control of trachoma has the acronym SAFE: **S**urgery for trichiasis and entropion, **A**ntibiotics for active disease, and **F**ace washing and **E**nvironmental improvement to reduce transmission. The antibiotic most commonly used for trachoma is topical tetracycline ointment, which needs to be applied daily or twice daily for six weeks. There is evidence that single dose oral azithromycin is more effective than topical tetracycline in clearing ocular *C. trachomatis* infection. However, more research is needed to establish optimal target groups and dosing frequency for azithromycin, and to improve strategies for delivery of the drug to endemic communities. Studies with kinetic quantitative PCR and investigation of weight-based dosing could make useful contributions.

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<sup>xxii</sup> defined by Lietman *et al.* as a prevalence of active disease in children at baseline of less than 35%

### 3. AIM AND OBJECTIVES

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The aim of this thesis is to provide evidence to assist the development of rational strategies for the use of azithromycin in trachoma control.

Specifically, the objectives are:

- (1) To determine, using quantitative PCR, the infective load of *Chlamydia trachomatis* in different age and gender groups in a community of approximately 1000 individuals before antibiotic treatment.
- (2) To measure, using quantitative methods, the effect of community-wide mass treatment with oral azithromycin 20mg/kg on the infective load of *C. trachomatis* in different age and gender groups.
- (3) To measure, using quantitative methods, the rate at which infection is re-introduced following community-wide mass treatment with azithromycin.
- (4) To determine the relative importance of different risk factors in predicting re-emergent infection following community-wide mass treatment with azithromycin.
- (5) To trace sources and routes of reinfection with *C. trachomatis* following community-wide mass treatment with azithromycin.
- (6) To determine the relationship between height and weight in a trachoma-endemic community in Tanzania, to investigate the feasibility of using height to guide the dose of azithromycin there.

## 4. RESEARCH BACKGROUND

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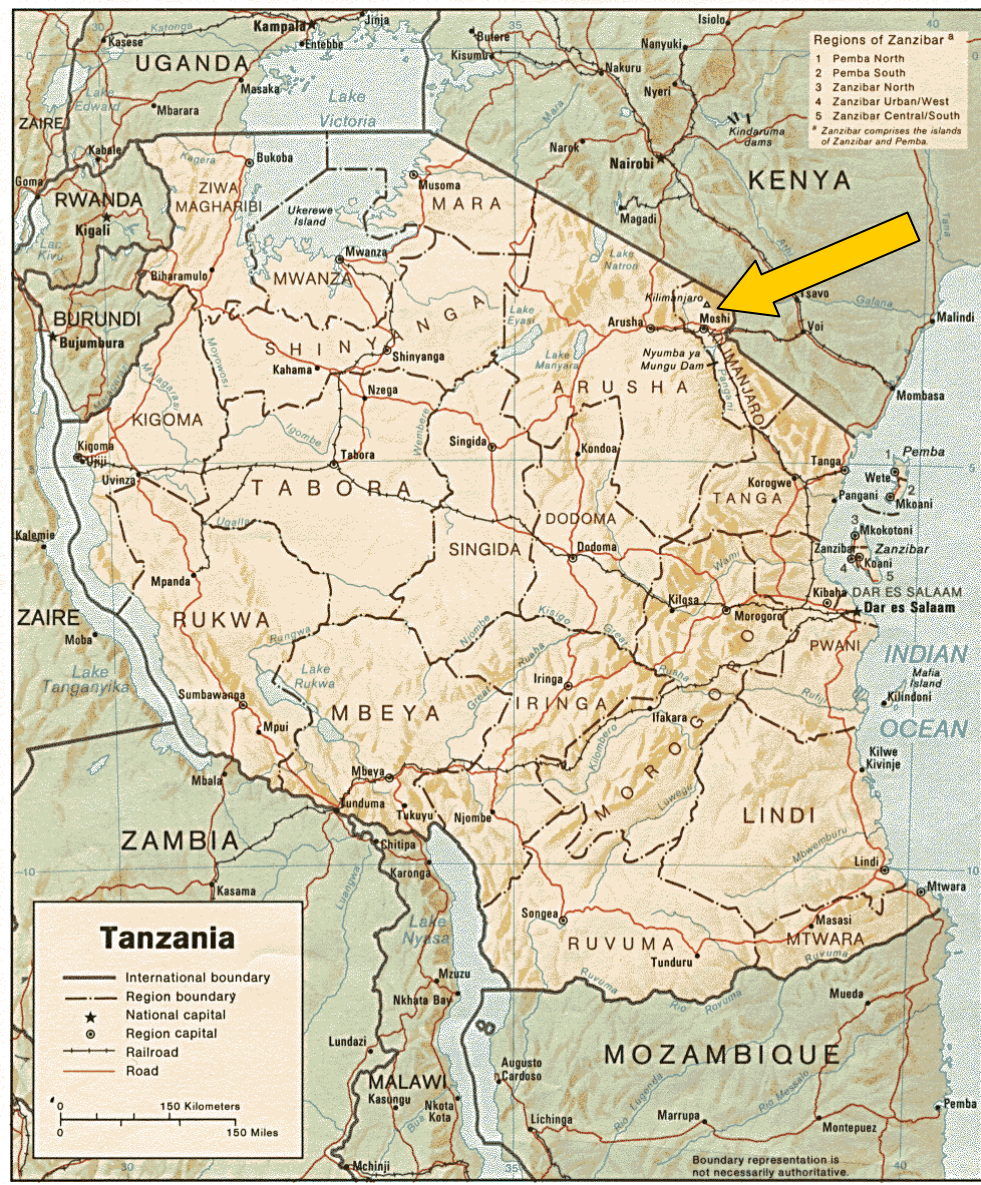
### 4.1 SETTING

Tanganyika became independent from Britain in 1961, and joined the Indian Ocean islands of Zanzibar and Pemba two years later to form the United Republic of Tanzania. Despite a radical programme of economic and social reform introduced by Julius Nyerere, the first president, Tanzania languishes near the bottom of the league table in indices of human development. In GDP terms, the country is the second poorest of the 173 for which data are available, with a per capita annual income of just PPP<sup>xxiii</sup> US\$523 in 2002 [527].

The mainland of Tanzania is divided into twenty regions, each of which is subdivided into districts. Rombo is an arid district of the Kilimanjaro region, lying just below Tanzania's northern border, on the eastern and northern slopes of Mt Kilimanjaro (Figure 4.1, p.100). In 2001, the total population of Rombo was estimated to be nearly 250,000 [528]. There are sixty-four villages, each of which has between 3,000 and 8,000 residents spread over a few square kilometres. Current sociopolitical structures are a legacy of ujamaa (socialism) and the 'villagisation' movement of the early 1970s [529]. Groups of approximately ten families living adjacent to each other are formed into a collective known as a balozi (plural: mabalози), a word also used for the man (or, less commonly, woman) who represents the group at village meetings. A group of seven to twenty mabalози creates a kitongoji, or sub-village, headed by a sub-village chairman. The village is more or less a product of map-making whim, with the nearest neighbours of a family living on the outskirts of a village just as likely to be in the next village as the home one. Villages are administered by a chairman, who is himself a balozi leader, and by an executive officer, a full-time employee of the government.

Rombo lies in the rain shadow of Mt Kilimanjaro. During the dry season, flow ceases in all the rivers whose courses wind down the northern slopes of the mountain. In all locations investigated in a 2002 hydrogeological survey of the district, substantial aquifers were only located more than a hundred metres underground, below a thick basaltic lava crust [530]. As a result, it is difficult and expensive to drill boreholes. Many villages therefore have no close water source. For some households, water collection during the dry season may involve a round trip of up to six hours.

Figure 4.1: Map of The United Republic of Tanzania. The location of Rombo District is indicated by the yellow arrow.



Villages are served by dirt roads, many of which are in a poor state of repair. Agriculture is the primary activity. Maize, beans and bananas are the staples, while bananas, sunflowers and coffee are commonly grown as cash crops. Most families keep small numbers of a variety of livestock. The typical house has walls constructed from a woven stick frame and mud, or roughly milled timber. Corrugated iron roofs are now more commonly seen than the traditional thatch.

<sup>xxiii</sup> In theory, at the PPP (Purchasing Power Parity) rate, PPP US\$1 has the same purchasing power in Tanzania as US\$1 has in the USA. It therefore represents a common currency through which economic indicators can be compared across countries [527].

## **4.2 ETHNICITY, CULTURE, EDUCATION, AND LANGUAGE**

The Chagga are the predominant ethnic group in Rombo. Despite being the first Tanzanian tribe to embrace the western economic and monetary systems after European colonisation, animal – and particularly cattle – ownership is still regarded as a symbol of wealth in Chagga society. An awareness of the risks posed by *wachawi* (sorcerers), combined with a fear of cattle thieves induces many to continue the traditional practice of keeping livestock inside the house overnight. In addition to the Chagga, there are small numbers of Masai, Kamba and Kikuyu living in Rombo. Most Rombo residents are regular attendees at a church of one of the Christian denominations active in the district. Many simultaneously maintain animist beliefs.

In Rombo, most residents speak one of a number of dialects of KiChagga as their mother tongue. However, the language of teaching in Tanzanian public primary schools is Swahili. Universal free primary education was introduced soon after independence [531], and despite the later re-introduction of and progressive increase in fees made necessary by low government revenues and high national debt repayments [532], most parents make determined efforts to ensure their children attend school. As a result, Swahili – originally a coastal language – is almost universally spoken by Tanzanians aged fifty years or less. Only a small proportion of people in the district know more than a few words of English.

## **4.3 HEALTH AND HEALTH SERVICES**

Basic health indicators for the Tanzanian population reflect the country's economic woes. In 2000, the national infant and under-five mortality rates were estimated at 104 and 165 per 1,000 live births respectively [533, 534], and the maternal mortality rate a frightening 1,100 per 100,000 live births [535]. UNAIDS estimate that up to 4.25% of men and 9.67% of women aged between 15 and 24 may be infected with HIV [536, 537]. Forty-seven percent of all Tanzanians are undernourished<sup>xxiv</sup> [538].

The medical services for Rombo District are provided by Huruma District Designated Hospital, which is managed and largely staffed by the Sisters of Our Lady of Kilimanjaro. Complications of malaria and HIV, lower respiratory tract infections, diarrhoeal disease, tuberculosis, malnutrition, trauma, and interventional obstetrics all make significant contributions to the case load. The hospital includes a respected eye

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<sup>xxiv</sup> Persons whose food intake continually falls below the minimum requirement, or whose food intake is continually insufficient to meet their energy requirements.

clinic staffed by up to four ophthalmic nurses. Huruma Hospital is about ninety minutes drive away from the Kilimanjaro Christian Medical Centre (KCMC) at Moshi, which includes a medical school (the second in Tanzania), and which functions as the zonal referral hospital for five northern regions of Tanzania. Specialist ophthalmologists from KCMC visit Huruma on a regular basis.

#### **4.4 TRACHOMA IN TANZANIA**

Trachoma has been reported in ten of mainland Tanzania's twenty regions. An estimated twelve million people live in endemic areas [539]. Some places are particularly badly affected: a survey in Kongwa District of central Tanzania in 1990, for example, found the prevalence of active inflammatory trachoma to be 60% in a sample of 3853 pre-school children. Eight percent of 484 individuals aged fifty-five or older in that survey had trichiasis and / or entropion [331].

A focus of trachoma is also found in Rombo, affecting perhaps 90,000 people. An informal survey conducted by Christoffel Blindenmission (CBM) in 1998 estimated the prevalence of active disease amongst Rombo schoolchildren to be approximately 20%.

#### **4.5 KAHE VILLAGE**

Kahe village was selected as the location to conduct the studies detailed in this thesis on the basis of its accessibility, relatively high prevalence of active trachoma identified during the CBM survey, and enthusiasm of village opinion leaders for their community's participation. It is reasonably typical of Rombo villages. It lies between latitude 3° 03' 00"S to 3° 04' 00"S and longitude 37° 36' 30"E to 37° 39' 20"E [530]. Its northern boundary is within a few kilometres of Tanzania's border with Kenya.

#### **4.6 COLLABORATING INSTITUTIONS**

This thesis was linked to a programme of trachoma research entitled 'Strategies for the control of blinding trachoma', which included studies in Rombo, in Kongwa District of Tanzania, and in The Gambia. The London School of Hygiene & Tropical Medicine (LSHTM) has responsibility for Rombo and The Gambia, while Johns Hopkins University, Baltimore, administers the Kongwa centre, within the structure of the long-running Kongwa Trachoma Project.

Huruma Hospital assisted the project by seconding to us a local ophthalmic nurse experienced in community eye work, by providing office and living space, and by

assisting with the recruitment and training of field staff. The Principal Medical Officer of Huruma and the author of this thesis were the joint local principal investigators for the research.

KCMC personnel were involved in formulating the research questions and preparing the original grant application, and assisted with project steering and administration. In addition, the freezers of the KCMC clinical laboratory were used for interim storage of samples prior to their shipment to the UK for analysis.

The many contributions made by individual collaborators, both from within these institutions and elsewhere, have been described in the Acknowledgements (p.11).

#### **4.7 FUNDING**

Work at all three sites of the 'Strategies for the control of blinding trachoma' programme is supported by programme grant number 059134 from the Wellcome Trust / Burroughs Wellcome Fund. In addition, the Edna McConnell Clark Foundation provided funds through its grant number 99100 for the initial phase of map-making, enumeration of the study population, and collection of baseline clinical samples in Rombo. From May 2002 until submission of this thesis in July 2003, the author's salary was supported through grant number 01-034 from the International Trachoma Initiative.



## 5. METHODS

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### 5.1 COMMUNITY CONSULTATION

Preliminary discussions were held with the Kahe village chairman and executive officer. A meeting of the village mabalozi and waganga (traditional healers) was then called, where opinions about priority community problems were sought, and possible participation in a trachoma research programme discussed. It was agreed that kikope (problems of the eyelashes) were a major cause of disability for the elderly, and a shared understanding was reached of how the proposed study might impact on this problem in Kahe and elsewhere. Enthusiasm for the study was expressed, and at a second meeting, community consent was given for the work to go ahead. Opinion leaders were asked to identify any potential shortcomings of the suggested conduct of the study, and to offer possible solutions.

### 5.2 MAPPING

During April and May 2000, field assistants visited each of the 1108 households in Kahe. Each kaya (house) was assigned a four-digit identity code. The first two digits in this code indicate the number of the balozi (01–90, pre-assigned on a list of the ninety Kahe mabalozi); the second two (01–99) indicate the arbitrary numbers assigned to each kaya. Kaya numbers were allocated sequentially as households were visited. The balozi / kaya (B/K) number of the house was recorded on an individual form for that house (Appendix 2, Survey form (a)). To prevent assignment of the same number to more than one household, all the households comprising any one balozi were mapped by a single field assistant. Each field assistant carried a list of all possible B/K numbers for their mabalozi, crossing off each number as it was used. Because the balozi leader's house was always the first visited in each balozi, that house always took kaya number 01.

On the same household form (Survey form (a)), a map of the compound was drawn showing the position of all rooms and other permanent structures. Each structure was assigned a code. Rooms in which people slept were given numbers. Store rooms, kitchens, bathrooms, latrines and animal enclosures had letter and number codes. The roof type (tin/straw/other/none) of each numbered room was recorded.

The field assistant then asked permission from the head of the household to paint the B/K number in a prominent position on the door of room one of the kaya. If

permitted, the room number was also painted on the external door of each sleeping room. Spray paint and a stencil were used to ensure that marking the doors for study purposes was not unsightly. Painted code numbers were maintained throughout the study by a field assistant who was resident in the village.

In July 2002, each household was re-visited to determine the location of the structures shown on the Survey form (a) for that kaya. A handheld Trimble GeoExplorer 3 GPS unit (Trimble Navigation Limited, Sunnyvale, CA, USA) was used. To allow later differential correction of field data, throughout the duration of field work, reference satellite data were continuously recorded by a Trimble Pro XR receiver, located at KCMC in Moshi, using Trimble Reference Station software. All household-level spatial data were collected as points. The position of each room was recorded by taking readings in front of its external door, and of latrines and animal enclosures by taking readings in front of the door or entrance point. The location of each household as a whole was determined by taking readings at the approximate centre of the compound, determined by eye. For each point of interest, at least six readings were taken and automatically averaged within the GPS unit to yield a single northing and easting. Because two years had elapsed since the original hand-drawn mapping exercise, there were occasional discrepancies between the compound maps and the location of structures on the ground at the time of GPS data collection. In most cases, the discrepancies involved the addition of extra rooms or structures. When features had been moved or demolished, it was often possible to determine their original locations: in these instances spatial data was recorded at those points [540]. Data for features where this was not possible were excluded from geographical analyses.

All GPS data were uploaded daily onto a laptop computer, using GPS Pathfinder Office 2.80 software (Trimble). Differential post-processing of the GPS field data against base station data was performed in Pathfinder Office.

### **5.3 SURVEY**

A house-to-house survey was performed, covering each household in Kahe. The purposes of this exercise were to compile a census list of all residents of Kahe village, and to collect village-wide data on clinical signs of trachoma and known and candidate risk factors for the disease. Personnel were divided into two survey teams, each comprising three field assistants, one examiner (Patrick Massae (an ophthalmic nurse),

or the current author), and a guide, who was either the village chairman or another senior balozi.

Participation in the survey was voluntary. Household heads and individual village residents were each given the opportunity to decide whether or not they wished to take part. They were assured that this would not affect their ability either to enter the study at a later date or to receive treatment for trachoma.

Balozi leaders were requested to notify household heads several days before the first scheduled visit of the survey team. Householders were asked to remain at home if this would not interfere with attendance at school or work. The village chairman's household was the first visited. Subsequently, the balozi leader's household was the first to be visited in each balozi.

The three field assistants in each team were responsible for household mobilisation and the collection of demographic information. One field assistant went to each kaya, informed all those present of the nature and aims of the study, answered questions, and invited householders to make themselves available for enumeration and examination. They then listed the members of the household (see below), obtained consent, and recorded data on household-level risk factors.

### **5.3.1 Definition of a household**

For the purposes of the study, a household was defined as the group of individuals sharing a building or group of buildings within a single shamba (plot of cultivated ground). In almost all cases this comprised a husband and wife, their dependant children, and any relatives, friends or household workers living with them. Where there was doubt, the balozi leader was asked to determine if a given group of individuals constituted one or more households.

### **5.3.2 Inclusion criteria for a household**

All individuals resident within the household were eligible for inclusion in the survey. Residency was defined as overnight stay for at least fifteen nights in the previous month, or recent arrival and intention to remain. Both males and females of all ages were invited to participate. Temporary visitors to a household were not included in the survey for that household.

In the case of individuals who divided their time between two or more households, the individual was asked to state in which household they had spent most

nights during the preceding month. This household was the one to which they were considered to belong. An exception to this rule was made for men who had more than one wife. The usual arrangement in Kahe is for each woman to live in her own household, with the husband regarded as the head of each of the households. To avoid duplicating data on these men, demographic details were recorded for them in each household in which they were the nominated head, but examination findings only for the house in which they were first seen. To ensure that this happened, the head of each household was asked before examination whether he had already had his eyes examined by a member of the trachoma survey team. If he had already been examined, the boxes for clinical findings were filled with the code '7', to simplify the identification of 'duplicate' individuals (see Table 5.1, p.109).

### **5.3.3 Consent**

Informed consent was obtained from each participating individual. Information sheets and consent forms were prepared in Swahili. One piece of paper was used to record consent for up to ten members of a household in tabular format (Appendix 2, Survey form (b)). Each individual was asked to sign his or her name in ink, or place their right thumb-print on the page against their name. For those aged under eighteen years, the consent of their parent or guardian was obtained.

### **5.3.4 Information recorded for each individual**

The first and last names, any aliases, date of birth, gender, ethnic group, room number slept in, highest level of formal education completed, and – if still at school – the current school attended, were recorded for each individual resident in the household. Details for up to ten householders were recorded on a single sheet (Appendix 2, Survey form (c)); if more than ten individuals were resident in the house, additional sheets were used and marked with the same household number. Table A3.1 (Appendix 3, p.209) contains the abbreviations and codes used on this form. Because these data represented our village census, demographic details were recorded to the fullest extent possible even for individuals who did not consent to examination, as well as for those who were not present at the time of the household visit.

### **5.3.5 Information recorded for each household**

In each kaya, an adult was asked to provide information about the household. A field assistant recorded answers to questions using a separate household form for each kaya (Appendix 2, Survey form (d)). Questions on this form sought data on the number of cattle, sheep, goats, pigs, ducks, chickens, dogs, pigs, rabbits and pigeons the family owned, and the number of cattle kept in the house or in the shamba that surrounded it. The time taken to walk from the house to the place from which water was collected in the wet season and in the dry season was recorded, as well as the number of times per week it was permitted to collect water in each season. Additionally, the informant was asked about the family's access to a latrine. Those that said that they had access were asked if the latrine was for the exclusive use of the family, or shared with another family. Because local government regulations require each family to maintain a latrine, respondents were reassured that our visit did not constitute a health inspection, and that all information given would be held in the strictest confidence.

If the family did claim access to a latrine, a field assistant asked permission to see it. He or she would then record (1) whether there was a beaten path to the door of the latrine; (2) whether the latrine was sufficiently well screened to ensure that the identity of a person inside the latrine could not be determined by a person walking along the nearest path that passed it; (3) whether there were flies (two or more or less than two) within the enclosure that surrounded the latrine; (4) the type of floor; and (5) using a torch, whether faeces were visible within the pit (Appendix 2, Survey form (d)).

### **5.3.6 Examination for facial cleanliness**

While preparing to examine each consenting individual for clinical evidence of trachoma, the examiner assessed the subject's face for the presence or absence of three signs of a 'dirty' face: eye discharge, nose discharge and fly-eye contact. Eye discharge was defined as pus or dried secretions around one or both eyes; nose discharge as discharge from the nose visible outside the margin of the external nares; and fly-eye contact as direct observation of one or more flies making contact with the lid margin (or tissue internal to the lid margin) during the time required to prepare for eye examination and examine the subject. Information about the face was recorded (Appendix 2, Survey form (c)), using the codes found in Appendix 3 (Table A3.2, p.211).

Villagers were not told in advance that facial cleanliness would be graded. Permission to withhold this information was obtained from the village chairman prior to the start of the survey.

### 5.3.7 Examination for trachoma

Both everted upper eyelids of each individual were examined using 2.5× binocular magnifying loupes and handheld torch. The WHO simplified trachoma grading scheme [200] was used, with three refinements.

‘P’ – in the context of trichiasis – was used to label cases in which five or more lashes touched the globe, distinguishing them from less severe trichiasis. This was to maintain consistency in notation between the three arms of the Wellcome Trust / Burroughs Wellcome Fund-supported ‘Strategies for the control of blinding trachoma’ study (see Section 4.6, p.102).

‘M’, for mild active trachoma, was added to protect the integrity of the diagnosis of TF. It reduces the temptation to classify fewer than five follicles, or small follicles, as TF, even though they do not quite meet the WHO simplified scheme criteria.

‘E’ was similarly created to protect the diagnosis of TS. The concept of “easily visible” [200] scarring is difficult to assess objectively, particularly in the blanched conjunctivae seen in many elderly subjects.

The full grading system used, including these additions, is presented in Table 5.1. (Final analysis was performed using the strict WHO simplified system definitions.)

Table 5.1: Trachoma grading scheme used (after Thylefors *et al.* [200])

Sign	Definition	Code
TT	<b>Trichomatous trichiasis</b> – at least one eyelash rubs on the eyeball, or evidence of recent removal of in-turned eyelashes.	Absent=0 One to four lashes=1 Five or more lashes=P Refused exam=5 Already examined elsewhere=7 Not recorded=9
CO	<b>Corneal opacity</b> – easily visible corneal opacity over the pupil, so dense that at least part of the pupil margin is blurred when viewed through the opacity.	Absent=0 Present=1 Refused exam=5 Already examined elsewhere=7 Not recorded=9

Table 5.1 (continued from previous page)

TF	<b>Trachomatous inflammation – follicular</b> – the presence of five or more follicles at least 0.5mm in diameter, in the central part of the upper tarsal conjunctiva.  One to four follicles (mild active trachoma, <i>not</i> ‘TF’)	Absent=0 Present=1 Unable to evert=4 Refused exam=5 Already examined elsewhere=7 Not recorded=9  Mild=M
TI	<b>Trachomatous inflammation – intense</b> – pronounced inflammatory thickening of the upper tarsal conjunctiva obscuring more than half the normal deep tarsal vessels.	Absent=0 Present=1 Unable to evert=4 Refused exam=5 Already examined elsewhere=7 Not recorded=9
TS	<b>Trachomatous scarring</b> – the presence of easily visible scars in the tarsal conjunctiva.  White patches that do <i>not</i> change with altered pressure but that are not ‘easily visible’ – presumed early scarring.	Absent=0 Present=1 Unable to evert=4 Refused exam=5 Already examined elsewhere=7 Not recorded=9  Early=E

### 5.3.8 Mopping up

Following completion of the first round of house-to-house visits, local schools were visited to see children who had not been present at their homes. Households from which demographic or examination data were still incomplete were then visited again, after notification of household heads. Lists of individuals still not examined were then compiled and given to mabalozi, who were asked to encourage listed people to attend one of a series of centralised examination sessions, or to bring word that the individuals concerned did not wish to participate. Balozzi leaders able to account for all individuals (examined, refused, absent) listed as resident in their balozzi were given a small cash sum (approximately £1).

### 5.3.9 Incidental findings

Upon identification of non-trachomatous eye pathology or non-ocular disease in study subjects (during fieldwork for the survey and the subsequent longitudinal study),

treatment or referral was offered to the patient whenever possible. In emergency cases, and for elective procedures booked at Huruma Hospital, patients were collected from Kahe and taken to hospital in the project vehicle.

## **5.4 LONGITUDINAL STUDY: BASELINE**

### **5.4.1 Selection of cohort**

Following completion of the survey, the kitongoji with the highest prevalence of active trachoma (Kahe Mpya – ‘New Kahe’; results by kitongoji are presented in Table 6.6, p.134) was invited to take part in the longitudinal study. This involved examination and conjunctival swab collection from all consenting sub-village residents before, and two, six, twelve, and eighteen months after, treatment of the entire village with azithromycin. The invitation to participate was accepted with enthusiasm by the kitongoji chairman and mabalozi of Kahe Mpya. The residents of Kahe Mpya are referred to hereafter as ‘the cohort’.

### **5.4.2 Numbering of individuals in the cohort**

Each individual registered at the survey as living in Kahe Mpya was assigned a unique six-digit identity (ID) number. All ID numbers used were multiples of eleven. Such numbers have the property that when any one digit is changed, or when two adjacent digits are transposed, the resulting number is no longer a multiple of eleven. This facilitated a simple check on the accuracy of data entry.

### **5.4.3 Sample labels**

Self-adhesive labels tolerant of high humidity and temperatures between –80 and +40°C (Tough-Tags™, Diversified Biotech, Boston, MA, USA) were used. The labels were laser-printed with sample codes prior to the commencement of fieldwork. A different code was used for each sample from each individual at each household visit. Seven digit numbers divisible by eleven were used. Each sample number was printed on two adjacent labels: in the field, one copy was fixed to the sample tube, the other to an individual data collection sheet on which the name and ID number of the study subject had been pre-printed (Appendix 2, Cohort form/00).

Any possible sample number was printed on a pair of ToughTags only once – no two pairs used at any stage of the study had the same number. The sample number was



therefore able to identify the individual from whom, the date on which, and the anatomical site from which the sample was taken.

#### **5.4.4 Database**

Demographic data obtained in the village survey were entered into a database purpose-built using Microsoft Access 97 SR-2. The report capability of Access was then used to pre-print subject ID cards, as well as individual forms for each of the baseline and follow-up visits of the longitudinal study.

#### **5.4.5 Baseline examination, swabbing, and treatment**

Baseline examinations for the longitudinal study were undertaken in July 2000. Everyone living within the cohort kitongoji was invited for examination, conjunctival swabbing, and treatment. Because of the number of personnel and volume of equipment required, a house-to-house strategy was not employed. Rather, permission was sought from Kahe Mpya mabalozi to see all individuals resident within their balozi, at their house, on designated days. Mabalozi were asked for assistance in mobilising the residents of their balozi. They were offered a small cash incentive (£2) to be awarded once each individual living in their balozi had been accounted for.

##### **(a) Baseline census update**

As indicated in Section 5.4.4, the forms used for the baseline cohort visit were pre-printed with the demographic data from the whole-village survey. Residence details were checked at the time of baseline cohort examination to ensure that they were still accurate. For individuals who had moved kaya or room since the survey, new details were recorded; a field assistant later visited them at home to confirm the B/K and room number of their new homes.

Any person living in Kahe Mpya at the time of the baseline round who had not been registered as being resident there in the survey had their details recorded on a blank form. Temporary visitors were not included. The household inclusion criteria for the longitudinal study were the same as those used for the whole-village survey.

##### **(b) Consent**

After reading the information sheet for the longitudinal study, answering questions, and checking the demographic data previously recorded for the individual, informed

consent for ongoing participation in the longitudinal study was requested. To confirm enrolment, written consent (in addition to the form that had been completed during the survey round) was obtained from each individual, or their parent or guardian.

### **(c) Examination and swabbing procedure**

Each consenting individual had a face photograph taken for ID card purposes (see below), and their face assessed for eye discharge, nose discharge, and fly-eye contact, as in the survey. The upper tarsal conjunctivae of both eyes was re-examined, using the same grading scheme (Table 5.1, p.109) that had been used for the survey. A sample was then taken from the upper tarsal conjunctiva of the right eye for *C. trachomatis* PCR, using a dry, sterile, Dacron polyester-tipped swab (“Puritan”, Hardwood Products Company, Guilford, Maine, USA). In testing endocervical specimens for *C. trachomatis* DNA by Amplicor PCR, dry Dacron swabs provide higher diagnostic sensitivity than do Dacron swabs swirled in specimen transport medium [282]. A standardised collection technique was employed, designed to minimise variations between individuals in the amount of material collected: the swab was held horizontally, and rubbed along the length of the conjunctiva four times, rotating it a quarter turn on each pass.

Individuals with nasal exudate visible outside the margin of one or both nares had a separate swab taken of this material for *C. trachomatis* quantitative PCR.

The same examiner (Patrick Massae) assessed and swabbed all subjects. In an effort to minimise cross-contamination of samples for PCR, and to prevent carry-over transmission of ocular pathogens from one subject to the next, he wore two pairs of latex medical examination gloves. The outer pair was removed and discarded after each subject had been seen, and the inner pair sprayed with isopropyl alcohol and allowed to air dry. A new pair of outer gloves was then donned. A new clean cotton-tip was used to evert the eyelid of each individual.

### **(d) Azithromycin**

Azithromycin was distributed to all consenting householders in Kahe Mpya during this visit, as described in Section 5.5 (p.114).

#### **5.4.6 Duplicate conjunctival swabs**

Prior to baseline swabbing, forty-seven individuals living in Kahe Mpya (5% of the 978 listed as being resident at the time of the survey) were selected at random (using a random number generator). Two eye swabs were to be taken from each of these individuals, if they were seen at baseline: the first for analysis with the rest of the cohort, and the second as a duplicate specimen to determine the reproducibility of the swabbing and laboratory techniques.

#### **5.4.7 Identity cards**

Face photos were taken of each individual recruited to the cohort. Each individual was asked to hold their consent/data form in front of their chest for the photo. The six-digit ID number of the individual was printed in large type on the top of this form. Film was developed in Moshi and printed as passport photos. ID cards were then prepared, displaying, in addition to the photo, the name, aliases, ID number, date of birth, and gender of the individual. Cards were printed using Microsoft Access.

Two laminated copies of each card were prepared. One was given to the individual at the two-month follow-up round in September 2000. The second copy was kept as a spare.

#### **5.4.8 Handling of samples collected during fieldwork**

Each swab taken was labelled with an adhesive sample label bearing a sample code, as described above, and placed on ice in a cold box in the field (4°C). At the end of each day swabs were put into a -20°C freezer at Huruma Hospital. After interim storage at KCMC, they were shipped to London on ice or dry ice. Details of the laboratory protocols appear in Section 5.7 (p.119).

### **5.5 TREATMENT**

In the cohort, antibiotics were offered at the baseline visit. After the baseline cohort visit, all non-cohort households in Kahe were revisited. For both groups, a single oral dose of approximately 20mg/kg azithromycin (to a maximum of 1g) was offered to every non-pregnant resident individual over one year old. In non-cohort mabalozi, distributors performed an initial round of balozi-to-balozi visits, after giving notice to residents that antibiotic would be available at their balozi leader's house on a specified

day. Several mop-up exercises were then undertaken in an attempt to make sure that no-one who wanted treatment missed out.

In accordance with national treatment guidelines, two tubes of 1% tetracycline eye ointment were given to children under the age of twelve months and to women who said they were pregnant, in place of azithromycin. Ointment was self-administered at home by the recipient or their carer. Recipients were taught the method of application, and instructed to apply the ointment twice a day for six weeks. Compliance with topical treatment was not monitored.

Azithromycin treatment was directly observed in every instance: drug was not given to third parties for onward distribution to family or friends. For village residents requesting treatment but unable to present themselves to receive it (the elderly, the ill, mothers with newborn children), home visits were made.

Those who refused clinical examination in the survey or enrolment in the cohort were still offered treatment. No charge was levied for azithromycin, tetracycline, or any other treatment.

#### **5.5.1 Weight measurement**

Subjects were first asked to remove their shoes and any heavy items that they carried in their pockets. Most were weighed to the nearest 0.1kg using electronic scales. Children unable to stand unsupported on the scales were weighed using spring scales. Individuals unable to be weighed (refused, too ill, too frail) had their weight recorded as '000.0' kg.

#### **5.5.2 Height measurement**

For adults and most children, height was determined to the nearest centimetre by asking the individual to stand erect and without shoes on a hard flat surface; a two-metre long wooden measuring stick marked from the base in centimetre increments was placed vertically at their back. A ruler held horizontally was used to bridge the distance between the subject's crown and the appropriate mark on the measuring stick. For children not yet able to stand, length from heels to crown was measured to the nearest centimetre by lying the child adjacent to the measuring stick. Adults or children with biomechanical disorders that prevented full extension, who refused, or who were too ill to be measured, had their height recorded as '000' cm.

### **5.5.3 Calculation of azithromycin dose**

Pre-prepared reference tables indicating required azithromycin dose-for-weight were used, to reduce the risk of errors in dose calculation. English and Swahili versions of these tables form Appendix 4 (p.212). For the small number of individuals who were unable to be weighed, a dose equal to that given to someone of similar age and appearance from the same balozi was dispensed.

### **5.5.4 Treatment records**

For treated individuals living in cohort mabalozis, details of body weight, height, planned dose, and administered dose were recorded in spaces on the baseline visit form (Appendix 2, Cohort form/00). For treated individuals living in non-cohort mabalozis, this information was recorded in boxes printed on the reverse side of the form used to gather demographic and clinical information during the whole-village survey (Appendix 2, Survey form (c)(back)). The codes used to record planned treatment and its success are shown in Table A3.3, Appendix 3, p.211). To avoid treating people more than once, to qualify for treatment, individuals had either to have been listed during the whole village census or baseline cohort round, or have their residence status confirmed by their balozi leader.

### **5.5.5 Trichiasis surgery**

Individuals identified as having trichiasis and/or entropion in one or both eyes were offered tarsal plate rotation procedures. Operations were performed by Patrick Massae, an experienced trichiasis surgeon well known to village residents, in the village dispensary, free of charge, and guides were available to assist patients to walk to the dispensary. Very frail patients were collected and returned in the project vehicle.

## **5.6 LONGITUDINAL STUDY: FOLLOW-UP**

### **5.6.1 Monthly census review**

Beginning thirty days after the first day of baseline examinations, a review of the cohort population was undertaken each month. Field assistants went house-to-house. At each household, one or more informants deemed to be reliable were asked to give information. For each person who had been registered as being resident in that household at the previous examination or review, confirmation was sought that the individual was still resident there, using the same criteria used for the original census in

the survey. Current sleeping room and current school attended were checked. The destination of those who had moved out of the house was recorded whenever this was known. Lastly, the informant was asked whether any new individuals were now living at that address.

At a subsequent face-to-face meeting, new arrivals to Kahe Mpya (from non-cohort households in Kahe, or from outside the village) and newborns were invited to enrol in the cohort. Demographic details were recorded, informed consent obtained, a face photograph taken for the preparation of an ID card, and a single swab collected from the everted upper tarsal conjunctiva of the right eye, for *C. trachomatis* PCR. A history was also taken to determine whether the individual had ever received azithromycin or topical tetracycline eye ointment, and if so, when. For those moving in from other mabalozi within the village, records from the treatment round were examined to help check the accuracy of this history. Ocular examination was not performed; nor was treatment for trachoma offered, with the following exception. At the time of the one-month census review in August 2000, treatment of non-cohort mabalozi in Kahe was just being completed (see Section 6.3, p.145). Individuals moving from non-cohort Kahe households to Kahe Mpya during the first month of the longitudinal study would therefore be unusually placed: had they stayed at their old residence, they would have been offered treatment. To avoid disadvantaging these people, all new arrivals to Kahe Mpya registered at the one-month census review were offered treatment, provided they had not already received it elsewhere.

The monthly visits were also used as a means of obtaining feedback from mabalozi and household leaders and enrolled individuals. In particular, the informant at each household was given the opportunity to identify concerns over any aspect of the conduct of the study.

When necessary, the Kahe Mpya sub-village map was updated. For newly formed households and newly constructed rooms added to existing households, B/K and room numbers were assigned. GPS readings were taken at a later date, as described in Section 5.2 (p.104).

### **5.6.2 Follow-up examination and swabbing**

Follow-up visits were undertaken at two, six, twelve, and eighteen months. At each of these time points, all available enrolled individuals then resident in Kahe Mpya were seen. Follow-up involved assessment of the face for eye discharge, nose discharge, and

fly-eye contact; examination of the everted upper tarsal conjunctivae of both eyes; collection of a swab of the upper tarsal conjunctiva of the right eye, using the standard technique; and collection of a swab of visible nasal exudates, if present.

Individuals who were registered as part of the cohort but who moved out of Kahe Mpya during the period of follow-up were deemed to be not part of the cohort for such time as they were living elsewhere. If such individuals subsequently returned to live in Kahe Mpya, they rejoined the cohort as 'old' individuals.

As at baseline, mabalozi leaders were offered small cash rewards for assistance in mobilising the population and the work involved in helping the field team account for each individual in their balozi.

### **5.6.3 Risk factors for re-emergent infection following treatment**

At the six, twelve, and eighteen month follow-up points, in addition to examination and swabbing as described above, each cohort individual who presented to be seen was asked to provide answers to the four questions:

1. In addition to working in your shamba, what sort of work do you do?
2. Where do you do this work?
3. Where have you travelled in the last six months?
4. Where have you travelled in the last month?

The same two interviewers, each of whom spoke both Swahili and the local Kichagga dialect (Kiusseri), undertook the task of asking these questions at all three of these follow-up points. Questions were read as written above, but further explanations and examples ("For example, have you been to a church outside the village recently? When?") were provided as necessary. Respondents were able to give an unlimited number of answers to each of the four questions. Family members were asked to provide information for young children and other individuals unable to speak for themselves. Job types and locations had pre-assigned numerical codes.

### **5.6.4 Re-treatment**

At the six, twelve, and eighteen month visits, individuals who had clinical evidence of active trachoma were offered two tubes of topical tetracycline eye ointment. As at baseline, treatment was given without charge. Compliance with tetracycline was not monitored. Treatment was not offered at the two-month visit, since it was felt likely that clinical signs present at that time were evidence of resolving rather than new

infection. Repeat administration of azithromycin was scheduled for twenty-four months.

## **5.7 LABORATORY METHODS**

### **5.7.1 Blinding**

All samples were tested blind to clinical assessment and to demographic information.

### **5.7.2 Qualitative PCR**

The Amplicor *Chlamydia trachomatis* qualitative PCR assay (Roche Molecular Systems, Branchburg, NJ, USA) was used for the first stage of sample analysis, as a screening test to select samples for quantification. Standard precautions against cross-contamination were employed. Benches, hoods and discard bins were cleaned with 5% Chlorox (sodium hypochlorite solution, Hays Chemical Distribution Ltd, Leeds, UK; stock contains >5% but <16% available chlorine) and 70% alcohol before and after each laboratory session. Sample racks, pipettes and other materials were similarly cleaned with 2% Chlorox and 70% alcohol. All sample pipetting was performed in a class II negative pressure isolation hood, using aerosol barrier tips. The amplification and detection areas were distinct, and separated from the sample preparation area by a self-closing door. PCR master mix was prepared in a second class II hood that was dedicated to this purpose.

Each swab was eluted by vortexing for ten seconds in 0.5mL Amplicor CT/NG lysis buffer in a 2mL polypropylene tube. Excess liquid was expressed from the swab against the inside of the tube, and 0.3mL Amplicor specimen diluent added. The lysed sample was held at room temperature for ten minutes, incubated at 95°C for ten minutes to inactivate inhibitors, and refrigerated overnight at 4°C. Heating and overnight refrigeration prior to amplification reduced the proportion of samples in which PCR was inhibited, and therefore the number of samples that needed two or more tests to obtain a result.

Amplicor CT/NG working master mix (MMX) was prepared by adding 100µL of CT/NG internal control to one vial of CT/NG master mix, recapping, and inverting the vial fifteen times. Working positive and negative controls were prepared freshly for each day, according to the manufacturer's instructions. The Amplicor NG-positive control (plasmid DNA containing *N. gonorrhoeae* sequences) served as the CT-negative



control. PCR was performed according to the manufacturer's instructions. The principles of this assay are described in Section 2.6.7 (p.53). In each well of the ninety-six well plate, 50µL of sample, working positive control or working negative control, and 50µL of MMX underwent thirty-five cycles of amplification on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, USA). Two positive and two negative controls were included in each plate. The final cycle had the same denaturation and annealing steps as the earlier cycles, but was held at 72°C for five minutes to ensure completion of chain extension.

In order to identify samples in which PCR was inhibited, we tested for amplification of both target (pCT) DNA and the MMX internal control (IC) sequence. Separate aliquots of the amplicon from each sample were added to two microwell plates, one of which contained a probe for the IC, and one of which contained a probe for the pCT target sequence. The protocol for ELISA and detection followed the manufacturer's instructions. Optical density (OD) was read at 450nm on an MRX microplate reader (Dynex Technologies, Chantilly, Virginia, USA). An OD of less than 0.200 in the internal control well was taken as evidence of inhibition of PCR. In these cases, an aliquot of the original lysed sample was diluted 1:5 with a 50/50 mixture of Amplicor CT/NG lysis Buffer and specimen diluent, then retested. If PCR was still inhibited at 1:5 dilution, a 1:10 dilution was prepared and amplified.

### **5.7.3 Selection and purification of samples for quantification**

All positive (optical density (OD)= 0.8 from the CT microwell plate) and equivocal ( $0.2 < OD < 0.8$  on two successive tests) samples were quantified. For each of these samples, 360µL of Amplicor preparation was processed through a QIAamp DNA Mini Kit (Qiagen Ltd, Crawley, UK), to purify and concentrate sample DNA, because the Amplicor buffers were not compatible with the quantitative PCR assay. QIAamp columns were used according to the manufacturer's instructions. QIAamp-purified DNA was resuspended in 50µL QIAamp AE elution buffer.

### **5.7.4 Quantitative PCR**

Real-time quantitative PCR was performed using the LightCycler (Roche Molecular Systems). The target for quantitative PCR amplification was a 123 base-pair fragment within the highly conserved constant domain 3 of *omp1*. Primer sequences (with melting temperatures,  $T_m$ ) were as follows:

CT1 – 5'-GCTGTGGTTGAGCTTTATACAGACAC-3' (T<sub>m</sub> 65°C)

CT2 – 5'-TTTAGGTTTAGATTGAGCATATTGGA-3' (T<sub>m</sub> 62°C)

To prepare standards for quantification, the target sequence was amplified and gel purified. Accurate estimation of the concentration of the stock so obtained was undertaken using PicoGreen<sup>®</sup> (Molecular Probes, Eugene, OR, USA) read at 502/523 nm in a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), analysed with SoftMax Pro (Molecular Devices). Absolute copy number of the stock was estimated according to the formula:

$$\text{number of molecules}/\mu\text{L} = \frac{(\text{fragment concentration (g}/\mu\text{L)})}{\text{fragment size(123 base pairs)} \times 660 \times 6.022 \times 10^{23}}.$$

Stock was serially diluted in sterile water containing 2 ng/μL Herring sperm DNA (Sigma, Poole, Dorset, UK) so that 4μL contained the required copy number of standard.

PCR master-mix was freshly prepared prior to the start of each run [238] by combining (per sample tested): 10μL QuantiTect™ SYBR Green PCR Master Mix (Qiagen; contains HotStarTaq™ DNA polymerase, QuantiTect SYBR Green PCR buffer, dNTP mix, SYBR Green I, ROX passive reference dye, and MgCl<sub>2</sub>), 1μL of 1μM solutions of each of the 3' and 5' primers and 4μL DNA/RNA-free water. 4μL of standard or re-suspended DNA from the QIAamp column was added to a glass capillary containing 16μL prepared PCR master-mix. For all but twenty of the quantified samples (see below), quantification was performed twice, on two independent 4μL aliquots, amplified on separate runs. Each run included six concentrations of standard (ten-fold dilutions of the stock solution from 10<sup>5</sup> to a single copy per capillary) and a negative or zero control. The profiles of fluorescence against cycle number of these seven capillaries were used to generate a standard curve. Thermal cycling was initiated with a fifteen minute, 95°C incubation step to activate the HotStarTaq polymerase.

The starting copy number present in each sample capillary was determined by comparison with the standard curve, using LightCycler Software v3 (Idaho Technology, Salt Lake City, Utah, USA).

### 5.7.5 Interpretation of PCR results

The sensitivity of the LightCycler assay was optimised so that it could amplify one copy of *omp1* per capillary (see Section 6.2.4, p.137). In spite of this level of sensitivity, Amplicor should be still more sensitive, for two reasons. Firstly, the 4μL of post-QIAamp resuspended DNA used for quantification is equivalent to 28.8μL of the original Amplicor preparation<sup>xxv</sup>, whereas 50μL of that preparation is used in the Amplicor assay. Secondly, Amplicor detects a multicopy plasmid rather than a single copy chromosomal gene, multiplying the volume advantage. Amplicor positive samples could therefore test negative in the LightCycler simply due to sampling variability. On this basis, a probabilistic estimate was obtained of the number of copies of *omp1* in Amplicor positive, LightCycler negative samples, as follows:

The volume of Amplicor preparation used in each Amplicor assay was  $vA=50\mu\text{L}$ . The Amplicor-preparation-equivalent volume used in each LightCycler capillary was  $vL=28.8\mu\text{L}$ . It is assumed that (1) the number of plasmids per organism was  $k=4$  [127]; (2) that samples were mixed homogeneously, and therefore that the number of copies of target DNA in each sub-sample would have a Poisson distribution; and (3) that both Amplicor and the LightCycler assay were 100% sensitive ([276] and Section 6.2.4(b) (p.137) respectively), so that if there were one or more copies of the target in the sub-sample, they would be reliably amplified and detected. With these assumptions, if the actual concentration of *omp1* in the Amplicor preparation is  $I$  copies per μL, the probability of having zero copies of *omp1* in a LightCycler capillary is

$$e^{-vLI}.$$

The probability of at least one plasmid in an Amplicor well is

$$1 - e^{-kvAI}.$$

The likelihood for an Amplicor positive, LightCycler negative sample is therefore

$$L = (1 - e^{-kvAI})e^{-vLI}.$$

To obtain  $\hat{I}$ , the maximum likelihood estimate of  $I$ , the equation is differentiated, and  $I$  set equal to zero, obtaining

$$\hat{I} = \frac{1}{kvA} \log\left(\frac{kvA + vL}{vL}\right).$$

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<sup>xxv</sup> For quantified samples, 360μL of the original Amplicor preparation is taken and concentrated (by purification and re-suspension) to 50μL in the QIAamp; 4μL of the 50μL is used in each LightCycler capillary. This is equivalent to using  $(4/50)*360 = 28.8\mu\text{L}$  of the Amplicor preparation

To obtain the estimated number of copies of *omp1* per swab, this is multiplied by 550µl. For the above values of  $vA$ ,  $vL$  and  $k$ , this yields 5.70 organisms per swab (or 0.30 per capillary).

If two capillaries are done, the probability of both testing negative is

$$\left(e^{-vLI}\right)^2 = e^{-2vLI},$$

so  $vL$  is replaced by  $2vL$  in the calculations. For such samples, the estimate is 4.12 organisms per swab (0.22 per capillary).

Two separate aliquots of most quantified samples were tested. When one or both aliquots were LightCycler positive, the geometric mean of the two replicates is presented here (including some with the value of 5.7 inferred for one of the replicates). Of all samples tested in the LightCycler, twenty (fifteen from the baseline round and five from the two-month follow-up) could only be tested once, due to low volumes of residual sample following optimisation of the assay.

### 5.7.6 Culture

Eye swabs collected in 2-SP medium were shipped on dry ice from London to the National Microbiology Laboratory, Health Canada, Winnipeg, Canada. HeLa 229 cells were grown in twelve-well tissue culture plates. Confluent twenty-four-hour old monolayers were treated for twenty minutes with 30µg/ml DEAE-dextran. Each conjunctival swab was vortexed for fifteen seconds with sterile glass beads and inoculated, in triplicate, onto the pre-treated HeLa cell monolayers. The inoculated cultures were incubated at 35°C in 5% CO<sub>2</sub> for one hour. Isolation medium, containing Minimal Essential Media, 10% fetal calf serum, 100mM glutamine and 1µg/ml cycloheximide, was then added to each well and the cultures were centrifuged at 1240×gravity for sixty minutes at 25°C. An additional 1mL of isolation medium was added to each well after centrifugation and the cultures were incubated at 35°C in 5% CO<sub>2</sub> for seventy-two hours. One monolayer culture from each specimen was stained for the presence of chlamydial inclusions using a fluorescein-conjugated monoclonal antibody specific for *C. trachomatis* (*C. trachomatis* culture confirmation kit, Syva Co., Palo Alto, USA). The remaining two monolayers were blindly passaged onto fresh HeLa cell monolayers. A specimen was considered negative if no inclusions were detected after ten blind passages.

## **5.8 DATA HANDLING**

A number of field assistants were given training in basic computer skills, and shown how to enter data using purpose-built ‘forms’ (data entry screens) that were constructed in a Microsoft Access database. Field assistants and the author undertook data entry during breaks between fieldwork.

Data collected on paper forms were double-entered into the database. Two versions of each base table and data entry form were constructed. The first-entry form directed the information to a first-entry table, and the second-entry form to a second-entry table. When a particular data set was complete, cross-checking Access ‘queries’ were run, matching corresponding entries in the two tables using ‘primary key’ field(s), and comparing the contents of other fields for mismatches between first and second entry. Lists of discrepancies were automatically entered into an errors table. Another query was used to create a table of records that had only been entered once, in either the first or second entry table. All errors were checked by hand by the current author, referring to the original paper data collection forms. Corrections were made to first or second entry base tables as necessary.

## **5.9 DATA ANALYSIS**

Analyses were performed using Stata 7.0 (Stata, College Station, TX, USA), Microsoft Access 2002 SP-2, and Microsoft Excel 2002 SP-2. Datasets were converted between the various formats required by these packages using Stat/Transfer 6.1 (Circle Systems, Seattle, WA, USA). Graphs were drawn in Stata and Microsoft Powerpoint 2002 SP-2. Maps showing the distribution of infected individuals by household at each time point were constructed in ArcView GIS 3.1 (Environmental Systems Research Institute, Redlands, CA, USA).

### **5.9.1 Analysis of clustering**

Analyses for spatial clustering of infected individuals were undertaken in SaTScan v3.0.4 (National Cancer Institute, Bethesda, MD, USA [541]). For each time point, case and population counts for each location and the geographical coordinates of these locations were the inputs. In each analysis, every household was considered as a possible location for the centre of a focus of elevated infection prevalence; a circular window was positioned at the geographical location of each house in turn, and its radius varied continuously from zero to that sufficient for the circle to enclose half the total

population examined at that time point. For each location and size of the scanning window, the number of cases within the window was compared to the expected number using a Poisson model, with adjustment for the uneven background population density [542]. For windows containing more cases than expected, the statistical significance of the departure from the model was evaluated in a Monte Carlo simulation with 999 replications [541].

### **5.9.2 An index for the average *omp1* load**

A summary statistic relating to the average load of infection was needed. For onchocerciasis, the community microfilarial load (CMFL) is used. It is calculated by taking skin snip microfilarial counts from all individual for whom the index is to be determined, adding 1 to each, calculating the geometric mean, then subtracting 1 from the result. Adding 1 to each value before calculating the geometric mean prevents the CMFL from being 0 whenever one or more of the microfilarial counts is 0. An analogous index could be used for ocular chlamydial load, but the method has a fundamental mathematical flaw: the value of the index is highly dependent on the units of the raw data. For example, the CMFL calculated using microfilariae per skin snip is markedly different from the CMFL for the same group of people calculated using microfilariae per milligram of skin. After investigating several alternative measures of central tendency, the CMFL approach was adapted by substituting the arbitrary value of 1 with the (equally arbitrary) smallest possible assay value, which at least has the advantage of removing the scale dependence of the index. As discussed in Section 5.7.5 (p.122), the smallest possible number of copies of *omp1* that can be determined using our laboratory methods is 4.12 per swab, or 0.22 per capillary. Throughout this thesis, the index, which will be referred to as the community ocular *C. trachomatis* load, or COCTL, has been quoted in copies of *omp1* per swab. Its confidence intervals were estimated in Stata, by bootstrap, with 10,000 replicates [543].

### **5.9.3 Risk factors for infection post-treatment**

Risk factors for infection at two, six, twelve and eighteen months after treatment were evaluated in separate logistic regression models for each time point. The dependent variable measuring infection was Amplicor status. As will be discussed in Section 6.5.3 (p.155), there were only nineteen Kahe Mpya residents who were Amplicor positive at two months, and progressively fewer positive individuals at six,

twelve and eighteen months. This meant that the number of explanatory variables which could be included in the analyses had to be restricted. Two confounders were included: age in years, measured as a continuous variable, and gender.

For the two and six month analyses, four sets of exposure variables were evaluated, chosen for their potential value in providing evidence for the A, F and E components of the SAFE strategy:

(1) Treatment status. Treatment codes entered on treatment record cards at baseline or one month (see Section 6.3, p.145) were summarised to create one binary variable for azithromycin and one for tetracycline eye ointment, each showing whether or not any of the corresponding treatment had been received at either time point.

(2) Presence or absence of eye discharge, nose discharge and fly-eye contact at the time of examination (one variable for each).

(3) Straight-line distance of the subject's residence (at the time of follow-up) to the Kahe Mpya standpipe, based on the northings and eastings determined by differentially-corrected GPS data.

(4) Access to a household latrine. This was used as a binary variable, disregarding whether or not the family had exclusive access to the latrine.

Logistic regression was undertaken using a step-up approach, beginning with an empty model. Each of the variables was fitted in turn; the most significant (determined by the likelihood ratio test) was added if its significance level was less than 0.05. This was then repeated, and the next most significant variable added if its significance level was less than 0.05. This process proceeded iteratively until the next most significant variable had a significance level of 0.05 or greater.

In fitting models for risks of being infected at twelve and eighteen months after treatment, the same method was used, but in addition, the effect of receiving tetracycline eye ointment at the previous round of examinations (see Section 5.6.4, p.118) was estimated.

## **5.10 ETHICS**

Research was conducted in accordance with the provisions of the World Medical Association Declaration of Helsinki, as revised at the 52<sup>nd</sup> General assembly, Edinburgh, Scotland, October 2000 [544].

### **5.10.1 Ethical considerations**

#### **(a) Equitable selection of subjects**

Kahe was chosen as the site for study because of its accessibility, relatively high prevalence of active trachoma, and proximity to trained local personnel and support facilities. Kahe Mpya was invited to become the cohort kitongoji because it was the sub-village of Kahe with the highest prevalence of active disease in the village survey. Regardless of cohort enrolment, all non-pregnant individuals over the age of six months in Kahe were offered antibiotic treatment according to national guidelines (Section 5.5, p.114), and, where appropriate, surgery for trichiasis (Section 5.5.5, p.116).

#### **(b) Potential benefit and the risk of harm**

Prior to the commencement of fieldwork, adult and child volunteers were asked to undergo eye examination and conjunctival swabbing in the manner described in Section 5.4.5(c) (p.112), to ensure that it would be tolerated. These volunteers reported that the swabbing procedure was mildly uncomfortable, but not painful.

Azithromycin was donated by the International Trachoma Initiative through the Tanzanian National Trachoma Control Programme, and was provided free of charge to recipients. Mass treatment was expected to dramatically reduce the prevalence of both ocular CT infection and clinical active trachoma in Kahe for some months after the intervention. The likely duration of this effect was difficult to estimate: this study was expected to be of value in providing data on which to base such predictions. Based on safety data and previous experience in its use on a community-wide basis in trachoma-endemic communities, a low incidence of serious adverse reactions was anticipated. Mass single dose antibiotic treatment poses potential risks for the selection of antibiotic-resistant bacterial strains; though the data do not appear in this thesis, studies conducted in Rombo in conjunction with those described here involved collection of data to monitor antibiotic resistance in *C. trachomatis* and *S. pneumoniae*.

Case-finding and subsequent treatment of individuals with trichiasis was expected to reduce the prevalence of trichiasis in Kahe to a very low level. Additionally, eye and systemic pathology detected as incidental findings during fieldwork were treated or referred as appropriate.

Training and experience in research methods and data entry was provided to a number of individuals in connection with their work on the study. Up to ten field workers were employed at various stages of the project.



Every effort will be made to ensure that the results of this work are published in peer-reviewed journals and used, where appropriate, to inform trachoma control policy. The research is therefore likely to benefit trachoma-endemic communities in the long term, including the one in which the investigations were conducted.

**(c) Consent**

Free and informed written consent was obtained from each participating individual, or their parent or guardian (Section 5.3.3, p.107; Section 5.4.5(b), p.112).

**(d) Confidentiality**

The confidentiality of personal information gathered in the course of the study has been maintained. Personal data will only be released to third parties with the express consent of the individuals concerned. When not being used for fieldwork, consent and data collection forms from the study are kept in secure locations in Rombo and London. Paperwork will be securely kept for five years following publication, and then destroyed.

Informed verbal permission to identify the study village in reports and published papers was granted without reservation by the Kahe village chairman and vitongoji leaders, on 30<sup>th</sup> March 2000.

**5.10.2 Ethical approval**

Ethical approval for these studies was obtained from both the LSHTM Ethics Committee (reference number 597 / 1999) and the KCMC Research and Ethical Clearance Committee.

A time line for the studies comprises Figure 5.1 (p.129).

Figure 5.1: Time line

	2000										2002												2003
	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	
Mapping																							
Survey																							
Cohort census review				↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	
Cohort examination																							
Cohort swabbing																							
Cohort treatment																							
Non-cohort treatment																							

\* Fifteen cohort individuals were treated at the one-month census review, as discussed in Sections 5.6.1 (p.116) and 6.3 (p.145).

\*\* Individuals with active disease at six months and twelve months were given two tubes of tetracycline eye ointment and asked to apply it to both eyes twice a day for six weeks (Section 5.6.4, p.118).

# 1. RESULTS

## 1.1 SURVEY

### 1.1.1 Census

As outlined in Section 5.3 (p.105), the population of Kahe village was enumerated as part of the survey, which was conducted between 11<sup>th</sup> April and 26<sup>th</sup> June 2000. At that time, field assistants listed a total of 5748 residents of Kahe village. Forty-five of these were identified as duplicate listings by examiners. The age and gender distribution of the 5703 'unique' village residents is shown in Table 6.1. Children aged fifteen years and under accounted for 54.2% of the village population. There were 2542 (44.6%) males and 3161 (55.4%) females. The preponderance of females over males was mainly found amongst sixteen to sixty year-olds, which probably reflects the tendency of men in this age bracket to leave the village to seek paid work.

Table 6.1: Age and gender distribution of the resident population, Kahe village, April – June 2000

Age (years)	Males (% of total village population)	Females (% of total village population)	Total (% of total village population)
0-	92 (1.6)	91 (1.6)	183 (3.2)
1-2	207 (3.6)	206 (3.6)	413 (7.2)
3-4	206 (3.6)	213 (3.7)	419 (7.3)
5-6	213 (3.7)	221 (3.9)	434 (7.6)
7-8	187 (3.3)	189 (3.3)	376 (6.6)
9-10	216 (3.8)	214 (3.8)	430 (7.5)
11-15	406 (7.1)	428 (7.5)	834 (14.6)
16-30	268 (4.7)	565 (9.9)	833 (14.6)
31-60	517 (9.1)	786 (13.8)	1303 (22.8)
61+	230 (4.0)	248 (4.3)	478 (8.4)
All	2542 (44.6)	3161 (55.4)	5703 (100)

The predominant ethnic group in Kahe is Chagga: 5674 (99.5%) of the 5703 residents identified themselves as belonging to this tribe. Seven (0.1%) said that they were Masai, six (0.1%) Kamba, and three (0.05%) Waimeru; the remaining thirteen (0.2%) belonged to a variety of other ethnic groups.

Of 4254 Kahe residents aged seven or more years (i.e., excluding pre-school age children), 1497 (35%) had received less than one year of formal education. The level of education completed is presented by age group in Table 6.2 (p.131) for males and in Table 6.3 (p.131) for females. Males over the age of thirty living in Kahe were significantly more likely than females over thirty to have completed at least one year of

school. In the 31-60 years age group, 406 of 481 males (84%), and 417 of 758 females (55%) had finished one or more years of primary school ( $\chi^2=114$ ,  $P < 0.00005$ ). In the 61+ years age group, the proportions were 59/224 (26%) and 7/243 (3%) for males and females respectively ( $\chi^2=53$ ,  $P < 0.00005$ ). In younger age groups, the proportions of males and females who had received at least some formal education were approximately equal (Table 6.2 and Table 6.3). Gender differences in successful completion of post-primary education were not significant.

Table 6.2: Level of formal education completed, by age group, males, Kahe village

Age (years)	Level of formal education completed (number, (%))						Total
	None	Any primary	Standard seven <sup>xxvi</sup>	Any secondary	Form six <sup>xxvii</sup>	Not known	
0-	92 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	92
1-2	207 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	207
3-4	206 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	206
5-6	213 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	213
7-8	170 (91)	17 (9)	0 (0)	0 (0)	0 (0)	0 (0)	187
9-10	115 (53)	101 (47)	0 (0)	0 (0)	0 (0)	0 (0)	216
11-15	24 (6)	382 (94)	13 (3)	2 (1)	0 (0)	0 (0)	406
16-30	9 (3)	259 (97)	172 (64)	15 (7)	0 (0)	0 (0)	268
31-60	83 (16)	433 (84)	266 (51)	9 (2)	4 (1)	1 (0)	517
61+	166 (72)	62 (27)	17 (7)	2 (1)	0 (0)	2 (1)	230
All	1285 (51)	1254 (49)	468 (18)	28 (1)	4 (0)	3 (0)	2542

Table 6.3: Level of formal education completed, by age group, females, Kahe village

Age (years)	Level of formal education completed (number, (%))						Total
	None	Any primary	Standard seven <sup>xxvi</sup>	Any secondary	Form six <sup>xxvii</sup>	Not known	
0-	91 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	91
1-2	206 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	206
3-4	213 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	213
5-6	219 (99)	2 (1)	0 (0)	0 (0)	0 (0)	0 (0)	221
7-8	175 (93)	14 (7)	0 (0)	0 (0)	0 (0)	0 (0)	189
9-10	107 (50)	107 (50)	1 (0)	0 (0)	0 (0)	0 (0)	214
11-15	29 (7)	399 (93)	21 (5)	0 (0)	0 (0)	0 (0)	428
16-30	26 (5)	539 (95)	466 (82)	21 (4)	1 (0)	0 (0)	565
31-60	352 (45)	433 (55)	296 (37)	6 (1)	1 (0)	1 (0)	786
61+	241 (97)	7 (3)	2 (1)	0 (0)	0 (0)	0 (0)	248
All	1659 (52)	1501 (47)	786 (25)	27 (1)	2 (0)	1 (0)	3161

At the time of the census, there were 1103 households. Some or all of the residents of 1099 were examined. No household-level data were available from four kayas in

<sup>xxvi</sup> The final year of primary education in the Tanzanian school system.

<sup>xxvii</sup> The final year of secondary education in the Tanzanian school system.

which people were examined. Together, these four were home to eleven individuals; for the other 1095 kayas, household details were complete. Residents in thirty (2.7%) of these 1095 had no access to a latrine, while 103 (9.4%) households shared a latrine with one or more other households, and 962 (87.9%) households had their own latrine. Only 300 (27.4%) households owned one or more cows. Given the importance that the Chagga attach to cattle ownership, the fact that less than a third of all households owned a cow can be interpreted as an indication of the poverty of the village. The mean reported time required to collect water in the dry season was 168 minutes (range 0–480).

### **1.1.2 Enrolment**

Of 5703 resident individuals, 5527 (96.9%) consented to enrolment in the survey, and were examined. Thirty-two people (0.6%) explicitly refused to participate. The remaining 144 (2.5%) were not seen at either the initial or follow-up house-to-house examination rounds, and did not attend the subsequent ‘special invitation’ examination sessions. Some of the 144 may have been working outside the village or had other commitments that prevented attendance for the entire three month duration of survey fieldwork, but most probably did not want to participate, and should therefore be considered refusals. The characteristics of individuals who were seen, who refused, and who did not attend is presented in Table 6.4 (p.133). Persons who did not attend (without explicitly refusing) had a significantly older mean age ( $z=4.38$ ,  $P<0.0001$ ), and were significantly more likely to have received secondary education ( $\chi^2=19.38$ ,  $P<0.0001$ ) compared to attendees. The other differences between the three groups in Table 6.4 were not statistically significant.

Of the 5527 people who presented, neither eyelid could be everted to permit examination of the upper tarsal conjunctiva in twelve (0.2%), because of short lashes, excessive tearing, inflexible tarsal plates, patient intolerance, or some combination of these factors. In a further fifteen individuals (0.3%), one upper tarsal conjunctiva could not be examined. Complete information on the presence or absence of conjunctival signs (TF, TI and TS) in both eyes is therefore available for 5500 people (99.5% of those enrolled in the survey; 96.4% of residents). Enrolment and examination data by kitongoji, including this information, is shown in Table 6.5 (p.133).

Table 6.4: Comparison of people seen and not seen in the survey, Kahe village, April-June 2000

Variable	Explicitly refused (n=32)	Did not attend (n=144)	Attended (n=5527 <sup>xxviii</sup> )
Mean age	28 years	29 years	23 years
Gender	69% male	54% male	44% male
Secondary education	0 (0%)	7 (5%)	55 (1%)
Household owns one or more cows	6 (19%)	36 (25%)	1710 (31%)
Household has own latrine	28 (88%)	130 (90%)	5020 (91%)
Mean reported time from household to water, dry season	172 minutes	162 minutes	168 minutes

Table 6.5: Number of residents, number examined, and number refused by kitongoji, survey, Kahe village, April – June 2000

Kitongoji	Explicitly refused	Did not attend	Examined for TT and CO; unable to evert one or both lids	Fully examined (% of total residents)	Total residents
Kahe Zamani	5	19	5	594 (95.3)	623
Ndong'e 'A'	3	27	5	773 (95.7)	808
Ndong'e 'B'	12	10	4	715 (96.5)	741
Kiura	0	7	3	581 (98.3)	591
Kahe Mpya	5	21	1	951 (97.2)	978
Ussarangei	5	39	4	927 (95.1)	975
Ture	2	21	5	959 (97.2)	987
Totals	32	144	27	5500 (96.4)	5703

### 6.1.3 Findings

The prevalence of active trachoma (TF and/or TI) is presented by kitongoji in Table 6.6 (p.134). Signs were considered present if seen in either the right eye or the left eye or both. In the analyses here, as elsewhere in this thesis, the strict WHO definition (TF=1 in Table 5.1, p.109) was used when classifying eyelids as showing 'TF' or 'no TF'. The prevalence of active disease ranged from 13.6% in Ndong'e 'A', to 27.6% in Kahe Mpya. Such heterogeneous distribution of disease is typical of trachoma, as was noted in Section 2.7.2 (p.70).

The main purposes of conducting the survey were (i) to identify the sub-village with the highest prevalence of active disease, and (ii) to obtain information about the prevalence and determinants of trachoma in Kahe. Only the first of these has relevance to the focus of this thesis: the study of ocular CT loads before and at intervals after mass

<sup>xxviii</sup> For secondary education, n=5523 (educational status not known for four individuals); for cattle ownership and latrine access, n=5516

antibiotic treatment. Further analyses of data collected in the survey are therefore not presented here.

Table 6.6: Prevalence of active disease in either or both eyes, by kitongoji, Kahe village, April to June 2000

Kitongoji	No. examined	Prevalence of active disease (%)
Kahe Zamani	594	102 (17.2)
Ndong'e 'A'	773	105 (13.6)
Ndong'e 'B'	715	146 (20.4)
Kiura	581	93 (16.0)
Kahe Mpya	951	260 (27.3)
Ussarangei	927	167 (18)
Ture	959	219 (22.8)
Totals	5500	1092 (19.9)

## 6.2 LONGITUDINAL STUDY: BASELINE

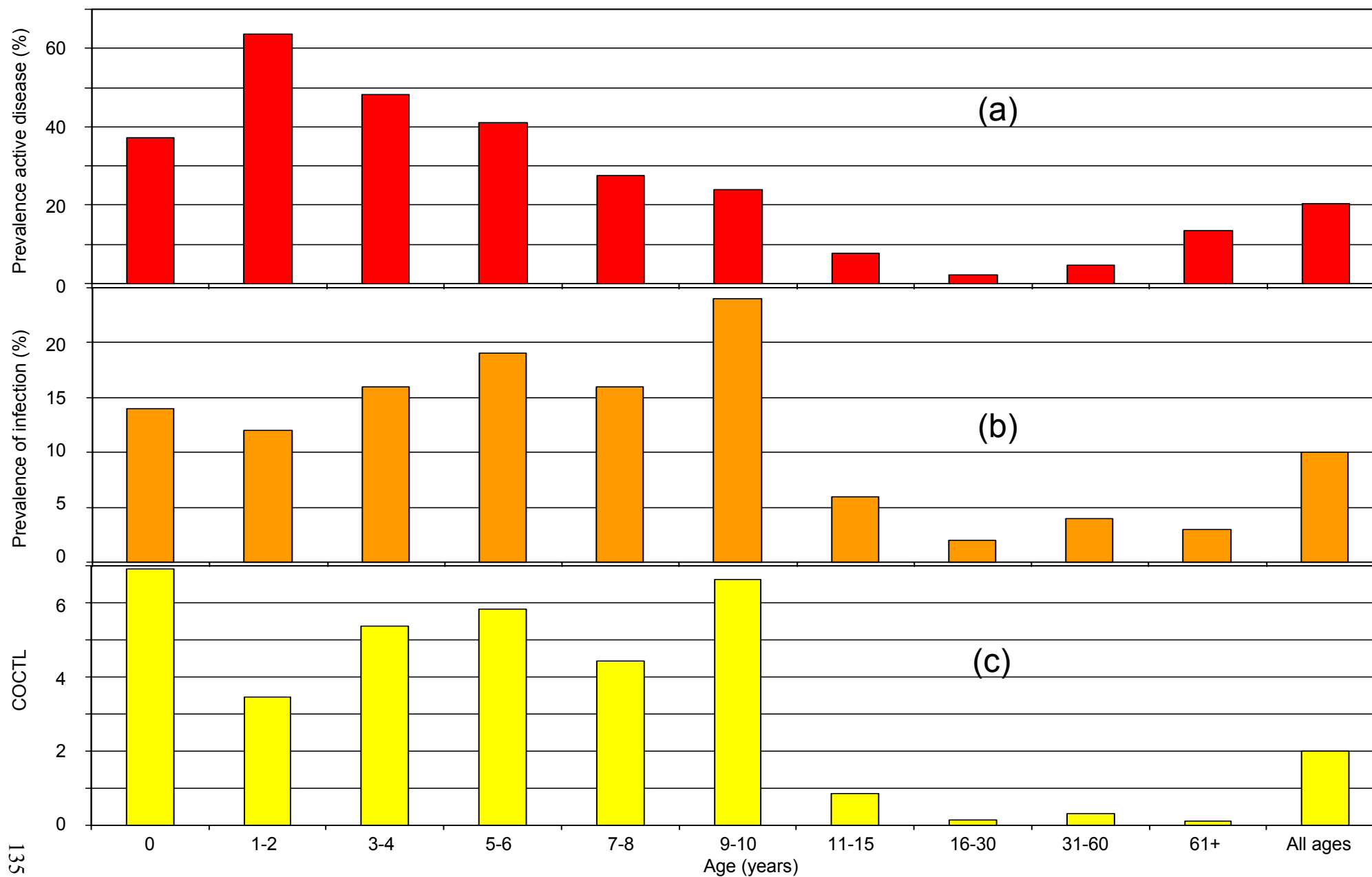
### 6.2.1 Enrolment

Of the seven vitongoji in Kahe, Kahe Mpya had the highest prevalence of active disease in the survey (Table 6.6), and was invited to take part in the longitudinal study. At the time of the survey, 978 people were resident in that subvillage (Table 6.5, p.133). The first survey visit to each Kahe Mpya household (at which time the data for that original census were collected) was made at some time (varying between households) between 10<sup>th</sup> May and 7<sup>th</sup> June 2000. For the longitudinal study, the baseline round of eye swabbing was undertaken between 11<sup>th</sup> and 24<sup>th</sup> July 2000. During the intervening period, three Kahe Mpya residents moved out to other vitongoji of Kahe, thirty-two moved out of the village altogether, two died, and the whereabouts of another became unknown to his family members. At the same time, thirty-eight new individuals were born or moved into Kahe Mpya (see Figure 6.8, p.153). Therefore, at the time of the baseline round, there were still a total of 978 (=978-3-32-2-1+38) individuals living in Kahe Mpya ('the cohort'). Of the 978, 956 (97.8%) were seen and examined at baseline, two (0.2%) were temporarily absent from the village, five (0.5%) explicitly refused, and fifteen (1.5%) did not attend for examination.

### 6.2.2 Clinical findings

The prevalence of clinical signs of active disease by age in the cohort at baseline is presented in Table 6.7 (p.136), and graphed in Figure 6.1 panel (a). The overall prevalence of active disease (TF and/or TI) in one or both eyes was 195/956 (20%). In

Figure 6.1: Prevalence of active disease (a), prevalence of conjunctival infection (b), and intensity of infection (COCTL) (c) by age group at baseline





[continued from end p.134] one to nine year-old children, the prevalence of active disease at baseline was 140/325 (43%) – more than double the WHO threshold for community mass antibiotic treatment. As expected, the prevalence of TF was highest in young children, peaking in the one to two year-old age group. TF became much less common with increasing age. The prevalence of conjunctival scarring, trichiasis and corneal opacity by age at baseline is shown in Table 6.8. Again as expected, and in contrast to active disease, TS, TT and CO were more frequently seen with increasing age. A five year-old boy was the youngest individual with TS; 45% of those aged sixty-one years or older had this sign. A total of fourteen of 956 individuals examined (nearly 1.5%) had TT. All fourteen were offered tarsal plate rotation procedures during August 2000 (see Section 5.5.5, p.116), as were sixty-two individuals with TT from the other six vitongoji. Forty-nine (64%) of the seventy-six patients presented for operation.

Table 6.7: Prevalence of TF, TI and active disease (TF and/or TI) in either or both eyes, by age, Kahe Mpya, baseline.

Age (years)	No. examined	TF (%)	TI (%)	Active disease (%)
0-	35	9 (26)	9 (26)	13 (37)
1-2	66	31 (47)	32 (49)	42 (64)
3-4	77	33 (43)	21 (27)	37 (48)
5-6	83	28 (34)	17 (20)	34 (41)
7-8	69	17 (25)	8 (12)	19 (28)
9-10	67	14 (21)	9 (13)	16 (24)
11-15	155	5 (3)	8 (5)	12 (8)
16-30	129	1 (1)	2 (2)	3 (2)
31-60	208	2 (1)	9 (4)	10 (5)
61+	67	0 (0)	9 (13)	9 (13)
All	956	140 (15)	124 (13)	195 (20)

Table 6.8: Prevalence of late stages of trachoma (TS, TT, CO) in either or both eyes, by age, Kahe Mpya, baseline.

Age (years)	No. examined	TS (%)	TT (%)	CO (%)
0-	35	0 (0)	0 (0)	0 (0)
1-2	66	0 (0)	0 (0)	0 (0)
3-4	77	0 (0)	0 (0)	0 (0)
5-6	83	1 (1)	0 (0)	0 (0)
7-8	69	3 (4)	0 (0)	0 (0)
9-10	67	2 (3)	1 (1)	0 (0)
11-15	155	4 (3)	0 (0)	0 (0)
16-30	129	6 (5)	0 (0)	0 (0)
31-60	208	61 (29)	8 (4)	4 (2)
61+	67	30 (45)	5 (7)	0 (0)
All	956	107 (11)	14 (1)	4 (0)

### 6.2.3 Swab collection

From the 956 individuals examined at baseline, 956 'primary' conjunctival swabs and sixty-seven swabs of nasal exudate were collected. In addition, forty-three 'duplicate' conjunctival swabs were taken. These were repeat conjunctival swabs from forty-three (91%) of the forty-seven individuals who had been randomly pre-selected for their collection (see Section 5.4.6, p.114): the other four selected individuals did not present to be seen. A total of 1066 swabs were therefore collected for PCR at baseline.

### 6.2.4 Reproducibility of laboratory results

#### (a) Reproducibility of the qualitative result

Of the forty-three individuals who had two conjunctival swabs taken for PCR at baseline, the primary and duplicate swabs gave the same result by Amplicor in forty-two (98%). The one discrepant result involved an Amplicor negative primary swab and an Amplicor positive duplicate. Two separate 4 $\mu$ L aliquots of the duplicate sample subsequently failed to produce a specific amplified product in the LightCycler, suggesting that infection was present at only a very low level.

#### (b) Sensitivity of the quantitative assay

Performing quantitative PCR on Amplicor positive baseline samples from all three sites of the 'Strategies for the control of blinding trachoma' study required a total of seventy-one separate LightCycler runs. Each run included one capillary assaying a 4 $\mu$ L aliquot of the most dilute standard (S1), which was prepared at a concentration of one copy of *omp1* per 4 $\mu$ L. Forty-six (65%) of the seventy-one S1 capillaries amplified successfully. Assuming homogeneous mixing of the standard solution, the number of copies of *omp1* found in repeated 4 $\mu$ L aliquots of S1 should have Poisson distribution, with a mean of 1 copy. On this basis, 37% ( $=e^{-1}$ ) [545] of S1 capillaries would be expected to contain zero copies of *omp1*, due to sampling variability. This is very close to the observed proportion (35%) of non-amplifying capillaries. It is therefore concluded that the Q-PCR assay was able to reliably amplify one copy of the 123 base-pair target sequence if it was present in the capillary, and that capillaries that did not amplify did not contain any target DNA. However, the sampling variability inferred for aliquots of the standard also applies to those taken from the samples: a capillary prepared from an Amplicor positive swab containing a very low CT load may have failed to test positive by Q-PCR simply because the 4 $\mu$ L aliquot sampled did not, by

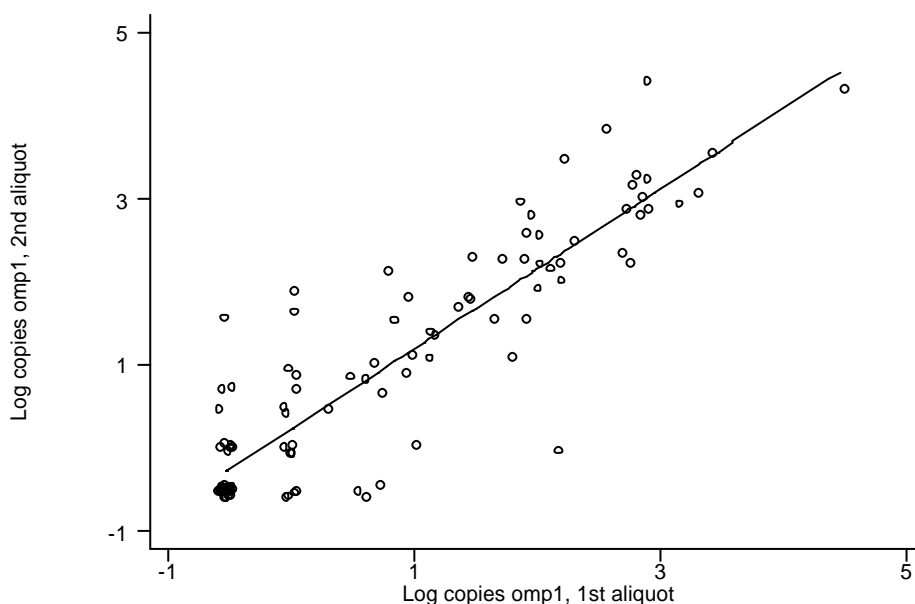
chance, contain any DNA. The derivation of a value for CT loads in such Amplicor positive, LightCycler negative samples is described in Section 5.7.5 (p.122).

### (c) Reproducibility of the quantitative result

Forty-three individuals had duplicate swabs taken. Five duplicates (12%) were Amplicor positive. As mentioned above, one of these positive duplicates was part of a discrepant pair (the primary swab was Amplicor negative, and therefore not quantified). For the other four individuals, the estimated number of copies of *omp1* determined by Q-PCR for the duplicate sample was within one log of that determined for the primary.

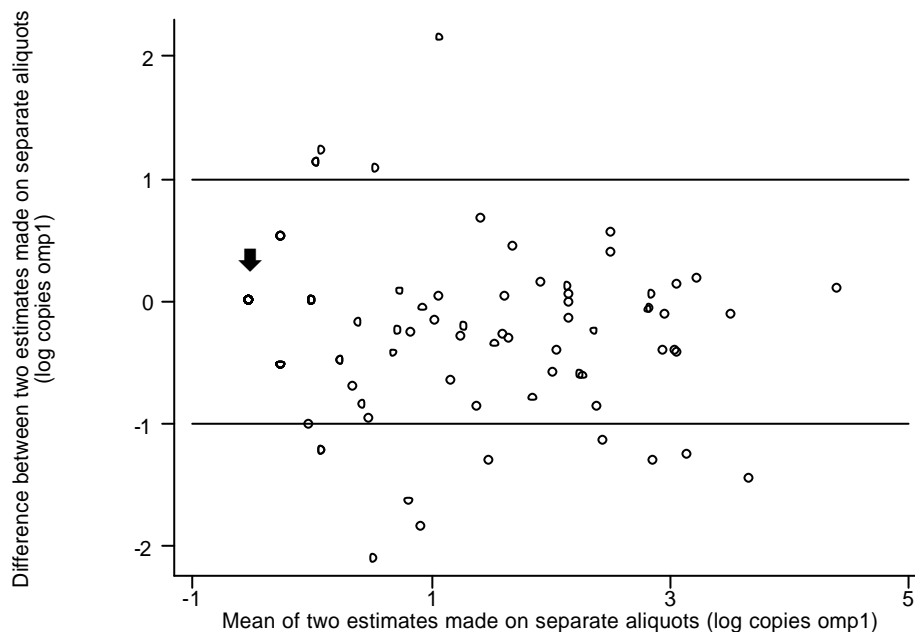
At baseline, there were 115 positive samples, including ninety-one primary conjunctival swabs (see Table 6.10, p.140), five duplicate conjunctival swabs and nineteen swabs of nasal exudate (see Table 6.11, p.141). In fifteen of the 115, the volume remaining after optimisation of the assay was not sufficient to allow two replicate 4 $\mu$ L aliquots to be tested. For the remaining 100 (87%), in Figure 6.2, the number of copies estimated per swab using the first aliquot is plotted against the number estimated per swab using the second aliquot. Values have been 'jittered' slightly, by the addition of a small amount of random noise, in order to separate points that would otherwise have been superimposed. The regression line of [second aliquot] quantified on [first aliquot] (i.e., using the results of the first aliquots to estimate the results of the second), fitted by least squares, is also shown.

Figure 6.2: Log<sub>10</sub> copies *omp1* determined for the second aliquot of each baseline Amplicor positive sample against log copies *omp1* determined for the first aliquot of the same sample



The slope (regression coefficient) of the line is 0.96 (95% CI 0.86–1.05); the correlation coefficient ( $r$ ) is 0.89 ( $P < 0.0001$  on 98 d.f.). However, as Bland and Altman [546] point out, the correlation coefficient measures the strength of the relationship between two variables, not the agreement between them, and it is extremely unlikely that two attempts to measure the same quantity would not be related. In Figure 6.3, the difference between the estimates made on the two aliquots of the same sample is plotted against the mean of those two estimates. The points between the two horizontal lines represent samples for which the difference between the first-aliquot and second-aliquot Q-PCR estimates was one log or less. Eighty-seven of the 100 samples with two replicates (87%) fell into this category. (Data points were not jittered for Figure 6.3; in this figure, therefore, a number of points are superimposed at  $(\log_{10}(0.3), 0)$ , indicated by the arrow,  $\blacktriangledown$ ).

Figure 6.3: Difference between the estimates of  $[\log_{10} \text{ copies } omp1]$  made on the two aliquots of each Amplicor positive sample at baseline, plotted against the mean of the logs of the estimates from those two aliquots



#### (d) Q-PCR positivity versus culture positivity

There were fifty-eight individuals with clinically active disease and an Amplicor positive swab at baseline. Culture swabs collected in 2-SP were available for fifty-six (97%). In this group, only those individuals who were Q-PCR positive (in addition to being Amplicor positive) were culture positive; all five of the individuals for whom both LightCycler replicates were negative were also culture negative (Table 6.9, p.140).

Table 6.9: Relationship between culture result, Q-PCR result and community ocular *C. trachomatis* load (COCTL) in subjects with signs of active trachoma and an Amplicor positive swab at baseline. The derivation of COCTL is explained in Section 5.9.2 (p.125); its confidence intervals were determined by bootstrapping, with 10,000 replicates.

Culture result		Q-PCR -	Q-PCR + on one or both replicates	
		No. subjects	No. subjects	COCTL (95% CI)
(+)	(+) on 1 <sup>st</sup> passage	0	11	11770 (2723 – 31967)
	(+) on 2 <sup>nd</sup> passage	0	17	5187 (1871 – 14375)
	(+) on 3 <sup>rd</sup> to 10 <sup>th</sup> passage	0	18	217 (87 – 621)
(-)	(-) on 10 passages	5	5	1095 (82 - 41405)
Total		5	51	

Compared to our quantitative PCR, then, the sensitivity of our culture technique would be 22% (11/51) if only one passage had been performed, 55% (28/51) if two passages had been performed, and 90% (46/51) when ten passages were performed.

### 6.2.5 Prevalence of infection

Ninety-one (10%) of 956 primary conjunctival swabs, five (12%) of forty-three duplicate conjunctival swabs and nineteen (28%) of sixty-seven swabs of nasal exudate tested positive by Amplicor for pCT DNA. Amplicor results by age and gender for the primary conjunctival and nasal exudate swabs are presented in Table 6.10 and Table 6.11 (p.141) respectively. None of the differences between males and females of the same age group were statistically significant (Fisher's exact test). The prevalence of conjunctival infection is plotted against age (males and females together) in Figure 6.1, panel (b) (p.135).

Table 6.10: Amplicor results by age and gender, conjunctival swabs, baseline

Age (years)	Amplicor + / number swabbed (%)		
	Males	Females	Total
0-	2/21 (10)	3/14 (21)	5/35 (14)
1-2	5/30 (17)	3/36 (8)	8/66 (12)
3-4	3/40 (8)	9/37 (24)	12/77 (16)
5-6	7/46 (15)	9/37 (24)	16/83 (19)
7-8	6/38 (16)	5/31 (16)	11/69 (16)
9-10	8/28 (29)	8/39 (21)	16/67 (24)
11-15	6/73 (8)	4/82 (5)	10/155 (6)
16-30	2/37 (5)	1/92 (1)	3/129 (2)
31-60	2/82 (2)	6/126 (5)	8/208 (4)
61+	1/27 (4)	1/40 (3)	2/67 (3)
All	42/422 (10)	49/534 (9)	91/956 (10)

Table 6.11: Amplicor results by age and gender, swabs of nasal exudate, baseline

Age (years)	Amplicor + / number swabbed (%)		
	Males	Females	Total
0-	0/1 (0)	1/2 (50)	3/15 (20)
1-2	1/6 (17)	1/6 (17)	
3-4	2/9 (22)	4/11 (37)	11/36 (31)
5-6	2/7 (29)	3/9 (33)	
7-8	2/5 (40)	0/2 (0)	4/11 (36)
9-10	1/1 (100)	1/3 (33)	
11-15	0/2 (0)	1/3 (33)	1/5 (20)
16-30	no swabs collected	no swabs collected	
31-60	no swabs collected	no swabs collected	
61+	no swabs collected	no swabs collected	
All	8/31 (26)	11/36 (31)	19/67 (28)

The prevalence of Amplicor positivity in swabs of nasal exudates was significantly higher than that in conjunctival swabs. Conjunctival swabs were collected from all consenting subjects, however, while nose swabs were collected only from individuals who had nasal exudate visible outside the margin of the nare (Section 5.4.5(c), p.112). The relatively high prevalence of infection in nose swabs may therefore simply indicate that individuals with nasal exudates were more likely to be infected with *C. trachomatis*, with the discharge being a response to that infection. This hypothesis is supported by the data: of sixty-seven individuals who had a nose swab taken at baseline (regardless of whether that swab was positive), sixteen (24%) had a positive baseline conjunctival swab, while only seventy-five (8%) of 889 individuals who did not have a nose swab taken had positive eye swabs ( $z=3.93$ ,  $P=0.00008$ ).

### 6.2.6 Intensity of ocular infection

Figure 6.4 (p.142) is a scatter plot of the estimated number of copies of *omp1* per swab by age and gender at baseline. Because the number of copies of *omp1* is displayed against a log scale, only data points relating to samples positive by Amplicor (copies *omp1*>0) are included. Values range from 4.12 copies *omp1* (the lowest possible value for the assay: see Section 5.7.5, p.122; seventeen individuals) to 491708 copies *omp1* (an eight month-old girl). Most individuals with high copy numbers were young. This is underscored by the differences in COCTL between age groups, shown in Figure 6.1 panel (c) (p.135), and tabulated in Table 6.12 (p.142).

Figure 6.4: Copies *omp1* per swab by age and gender at baseline for subjects with an Amplicor positive swab ('o'=male, '+'=female)

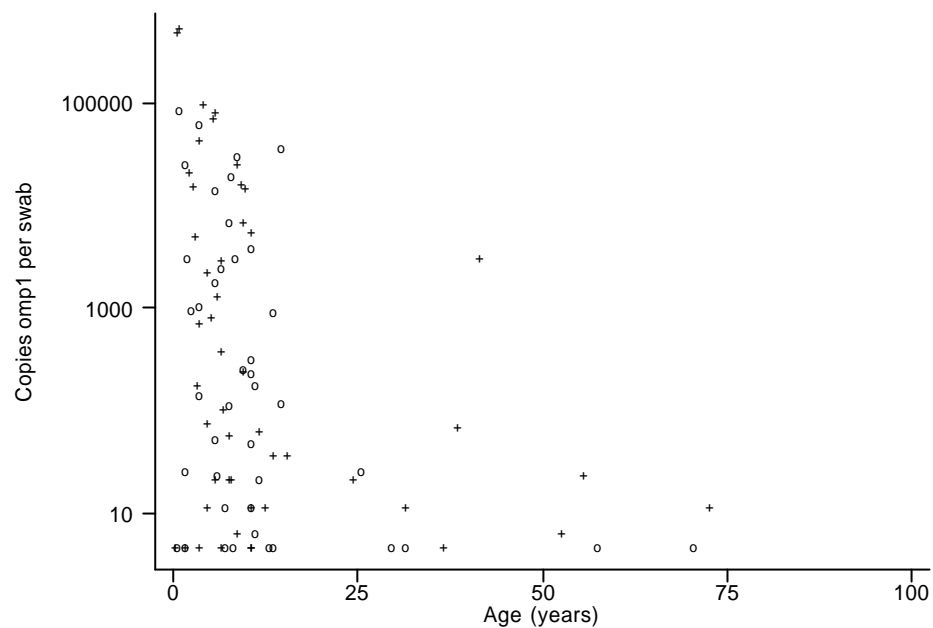


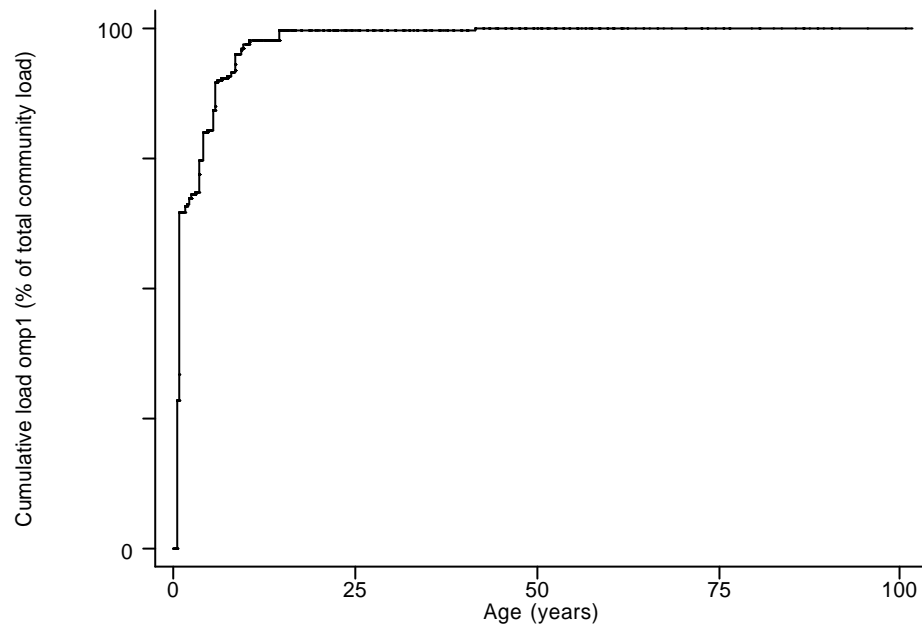
Table 6.12: COCTL by age group and gender at baseline. Both Amplicor positive and Amplicor negative individuals are included. Confidence intervals determined by bootstrapping, with 10,000 replicates.

Age (years)	COCTL (95% CI), <i>n</i>		
	Both genders	Males	Females
0-	6.9 (0.3 – 35.0), 35	2.7 (0.0 – 24.4), 21	18.7 (0.2 – 599), 14
1-2	3.5 (0.9 – 9.2), 66	4.8 (0.8 – 19.2), 30	2.5 (0.1 – 12.4), 36
3-4	5.4 (1.9 – 12.6), 77	2.4 (0.4 – 9.4), 40	10.1 (2.6 – 34.5), 37
5-6	5.8 (2.4 – 13.1), 83	3.2 (0.8 – 9.3), 46	10.5 (2.8 – 33.7), 37
7-8	4.4 (1.5 – 10.7), 69	6.2 (1.4 – 22.0), 38	2.7 (0.5 – 10.2), 31
9-10	6.6 (2.7 – 15.3), 67	6.7 (2.1 – 19.6), 28	6.6 (1.6 – 21.2), 39
11-15	0.8 (0.3 – 1.9), 155	1.3 (0.3 – 3.9), 73	0.4 (0.1 – 1.0), 82
16-30	0.1 (0.0 – 0.4), 129	0.3 (0.0 – 1.1), 37	0.1 (0.0 – 0.4), 92
31-60	0.3 (0.1 – 0.7), 208	0.1 (0.0 – 0.3), 82	0.5 (0.1 – 1.2), 126
61+	0.1 (0.0 – 0.4), 67	0.1 (0.0 – 0.6), 27	0.1 (0.0 – 0.9), 40
All	1.99 (1.44 – 2.67), 956	2.0 (1.2 – 3.0), 422	2.0 (1.3 – 3.0), 534

Another way of looking at the data is to consider the cumulative load of ocular *C. trachomatis* with increasing age. The total estimated number of copies of *omp1* found in all baseline swabs was 1,578,724. Figure 6.5 (p.143) is a plot of the cumulative number of copies of *omp1* against age in years at baseline, with cumulative load (on the vertical axis) shown as a percentage of that total community load. Fifty percent of the total community swab-load of *C. trachomatis* DNA was found in samples from children below nine months of age, and over 90% was found in samples from children aged

below six years and eight months. The importance of this result, and the contribution to it by two heavily infected infants, will be considered in the discussion.

Figure 6.5: Cumulative load of *omp1* found in conjunctival swabs against age at baseline



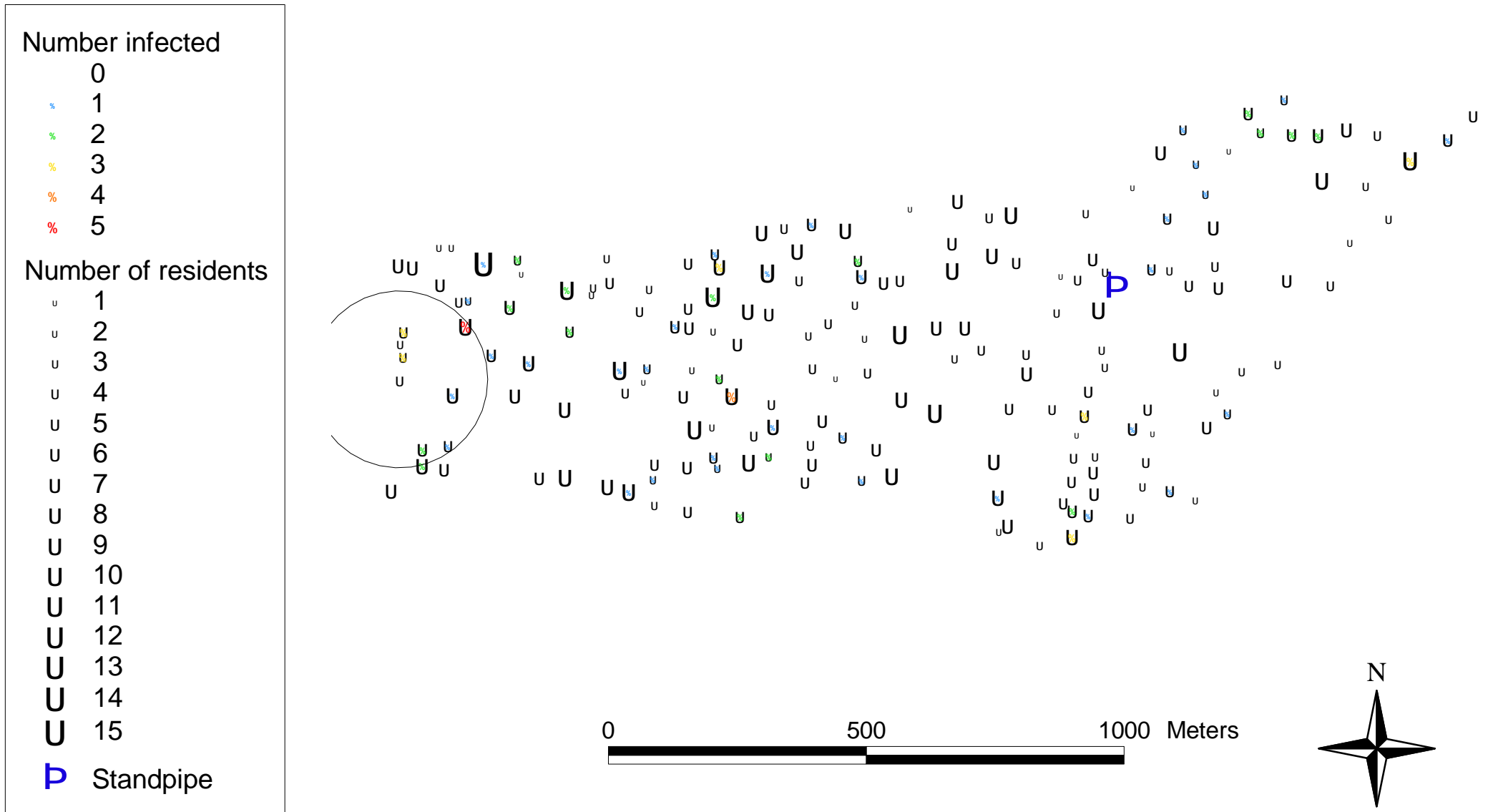
The wide confidence intervals for the COCTLs (and resulting lack of statistically significant differences in this index between males and females in each age group) in Table 6.12 (p.142) could be interpreted as stemming from the relatively narrow age bands used. Further analysis with data combined over broader age categories was therefore undertaken. An example is shown in Table 6.13. This shows gender-specific COCTLs separately for those above and below the age of ten, (based on the observation that the COCTL was markedly lower in those aged eleven and above than in the age groups up to ten years – see Figure 6.1 panel (c), p.135). The differences in COCTL between males and females, using these or other age categories, are not significant.

Table 6.13: COCTL for males and females, stratified into groups above and below the age of ten years, at baseline. Confidence intervals determined by bootstrapping, with 10,000 replicates.

Age (years)	COCTL (95% CI), <i>n</i>	
	Males	Females
<10	3.62 (1.88 – 6.49), 186	7.26 (3.83 – 13.13), 174
10 or more	0.94 (0.44 – 1.69), 236	0.42 (0.19 – 0.75), 360



**Figure 6.6: Distribution of Amplicor positive individuals (by household) at baseline**



### 6.2.7 Spatial distribution of ocular infection

Figure 6.6 (p.144) is a map showing the distribution of infected individuals by household at baseline. The number of people living in each house is represented by the size of the open (black-edged) square. The number of Amplicor positive individuals in the house is shown by the size and shade of the contained colour splash. The ‘most likely’ clustering<sup>xxix</sup> of infected individuals has a radius of nearly 180 metres, and is located within the circle drawn at the western boundary of the subvillage. This area contained seventeen cases; the expected number, assuming homogeneous distribution of cases amongst all swabbed cohort individuals (the null hypothesis), was 4.85 ( $P < 0.006$  based on 999 Monte Carlo replicates). None of the possible secondary clusterings identified by SaTScan were statistically significant (Appendix 5, p.213).

## 6.3 TREATMENT COVERAGE

Each non-pregnant member of the cohort above the age of twelve months who presented at baseline was offered a single oral dose of azithromycin 20mg/kg, given immediately after swabs were collected. As described in the methods section, women who said they were pregnant and infants less than one year old were not given azithromycin, but instead offered two tubes of tetracycline. All but one of the 956 cohort individuals who presented accepted either azithromycin or tetracycline. One sixty-year-old woman refused treatment because she had taken traditional medicine on the day of our visit, and did not want its effect to be diminished. Overall, for Kahe Mpya (the cohort), therefore, treatment coverage was 955/978, or 97.6%. Data are shown in Table 6.14 (p.146) for males, and Table 6.15 (p.146) for females. One individual mistakenly received treatment twice: once at home, and once at school.

In the other sub-villages of Kahe, it was not logistically possible to update the results of the original census to the same degree of exactness as was attempted for the cohort. Individuals who were not registered in the survey but who were resident at the time of drug distribution were listed on treatment record cards as new individuals, and offered antibiotics. However, no attempt was made to determine how many people

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<sup>xxix</sup> The term ‘clustering’ has been preferred to ‘cluster’ in this thesis, because if risk of being infected is in some way dependent on distance to – for example – other infected individuals, or a site where flies breed, then it is likely that that risk simply fades with increasing distance, rather than having a defined boundary [547]. However, to look for foci of elevated prevalence, it is necessary to delineate areas between which prevalence can be compared. SaTScan uses circular windows, identifying the location of each putative high prevalence area using the radius of the circle that delimits it, and the northing and easting of its centre. The focus of elevated prevalence has therefore been drawn as a circle in Figure 6.6, and its radius noted here, whilst recognising that a sudden transition between elevated risk of ocular CT infection and reduced (or background level) risk is unlikely in the real world.

Table 6.14: Treatment coverage for males by age group, cohort. The denominator for coverage in each cell is the number of male residents of that age living in the sub-village at the time of treatment, not the number examined.

Age (years)	Total residents	Number (% of residents in that age group)			
		Treated with azithromycin	Given tetracycline ointment	Did not present	Explicitly refused treatment
0-	21	9 (43)	12 (57)	0 (0)	0 (0)
1-2	30	30 (100)	0 (0)	0 (0)	0 (0)
3-4	40	40 (100)	0 (0)	0 (0)	0 (0)
5-6	46	46 (100)	0 (0)	0 (0)	0 (0)
7-8	38	38 (100)	0 (0)	0 (0)	0 (0)
9-10	29	28 (97)	0 (0)	1 (3)	0 (0)
11-15	74	73 (99)	0 (0)	1 (1)	0 (0)
16-30	43	37 (86)	0 (0)	6 (14)	0 (0)
31-60	90	82 (91)	0 (0)	8 (9)	0 (0)
61+	27	27 (100)	0 (0)	0 (0)	0 (0)
All	438	410 (94)	12 (3)	16 (4)	0 (0)

Table 6.15: Treatment coverage for females by age group, cohort. The denominator for coverage in each cell is the number of female residents of that age living in the sub-village at the time of treatment, not the number examined.

Age (years)	Total residents	Number (% of residents in that age group)			
		Treated with azithromycin	Given tetracycline ointment	Did not present	Explicitly refused treatment
0-	14	10 (71)	4 (29)	0 (0)	0 (0)
1-2	36	36 (100)	0 (0)	0 (0)	0 (0)
3-4	38	37 (97)	0 (0)	1 (3)	0 (0)
5-6	37	37 (100)	0 (0)	0 (0)	0 (0)
7-8	32	31 (97)	0 (0)	1 (3)	0 (0)
9-10	39	39 (100)	0 (0)	0 (0)	0 (0)
11-15	83	82 (99)	0 (0)	1 (1)	0 (0)
16-30	95	81 (85)	11 (12)	3 (3)	0 (0)
31-60	126	114 (90)	12 (10)	0 (0)	0 (0)
61+	40	39 (98)	0 (0)	0 (0)	1 (2)
All	540	506 (94)	27 (5)	6 (1)	1 (0)

[continued from end p.145] registered at the survey had died or moved out of the village in the interval between the survey and treatment visits. Of the 4732 individuals living in 'non-cohort' mabalozi (NCBs) in April through June 2000, 3921 (82.9%) presented for and received treatment in July or August 2000. An additional 125 'new' individuals were treated. Ignoring out-migration, deaths, and new-borns or new arrivals who did not present, then, the approximate treatment coverage for NCBs was  $(3921+125)/(4732+125)$ , or 83.3%. Three NCB individuals managed to receive treatment twice by presenting at different locations on two different days.

Treatment of NCB residents was undertaken from the 27<sup>th</sup> July to the 10<sup>th</sup> August 2000. The final day of drug distribution in Kahe was therefore thirty days after the first (11<sup>th</sup> July 2000: see Section 6.2.1, p.134). This cut-off date was imposed in order to simulate, as closely as possible, synchronous mass community treatment. Between the end of the baseline cohort round and the 10<sup>th</sup> August, eighteen new individuals were born or moved into Kahe Mpya. Fifteen of the eighteen requested antibiotic from the treatment teams, and were treated.

The total number of individuals treated in the village of Kahe was therefore  $(955+3921+125+15) = 5016$ . If we again discount the reservations about the reliability of the denominator for the NCBs (discussed above), the overall treatment coverage for the village was  $5016/(978+4732+125+18) = 85.7\%$ . These entire village treatment data are shown in Appendix 6: for males in Table A6.1 (p.228) and for females in Table A6.2 (p.229).

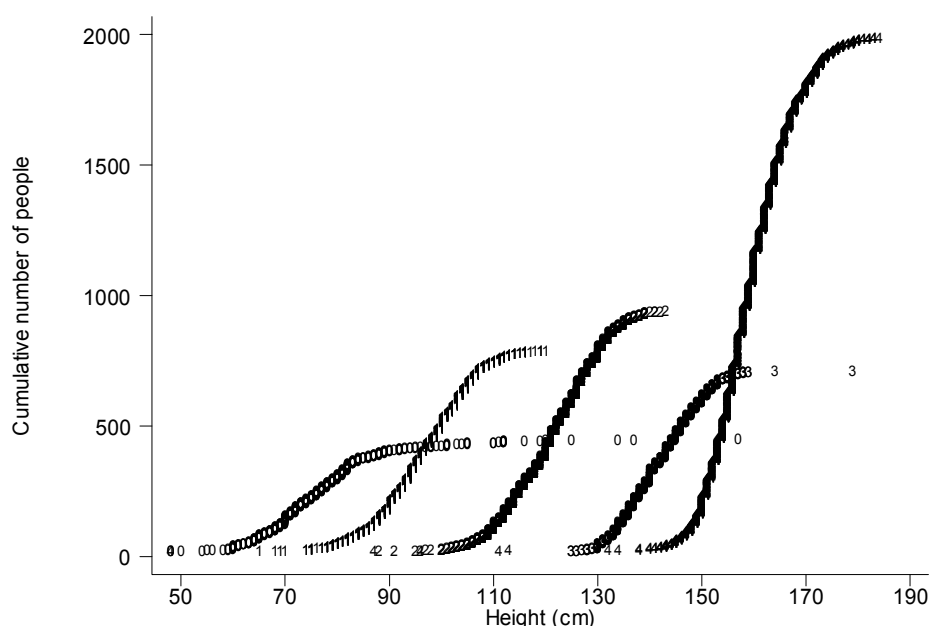
#### **6.4 HEIGHT AND WEIGHT DATA**

Of 5016 people treated, there were thirty-five individuals (0.7%) for whom height had not been measurable (and therefore recorded as 000cm: see Section 5.5.2, p.115); for three of the thirty-five, weight had also not been measurable (see Section 5.5.1, p.115). Another five individuals (0.1%) had weight recorded as being below one kilogram, despite their height being listed at sixty centimetres or more. These forty individuals, plus the eighty-one pregnant women and 117 infants (not given azithromycin) have been excluded from the analysis that follows, which is therefore based on the records of 4778 azithromycin-treated people (95.3% of those treated; 83.8% of the best-estimate village population). Azithromycin dose was determined by weight. The minimum weight required for tablets to be offered was 10.0kg; people below this weight were offered azithromycin suspension. A total of 4350 people were given one or more azithromycin tablets. Summary statistics for the heights of people in each weight-based tablet dosing category comprise Table 6.16 (p.148). These distributions are shown graphically in Figure 6.7 (p.148).

Table 6.16: Recorded heights of subjects in five weight-based tablet dosing categories

No. of tablets (by weight)	No. of people	Height (cm)				
		Minimum	25 <sup>th</sup> percentile	Median	75 <sup>th</sup> percentile	Maximum
0	428	48	70	76	82	157
1	770	65	90	97	103	120
2	920	88	115	121	128	143
3	691	125	136	142	147	179
4	1969	48	154	159	164	184
Overall	4778	48	109	140	157	184

Figure 6.7: Cumulative height distributions of azithromycin-treated Kahe residents, by weight-based dosing category. Symbols used to construct each curve represent the number of tablets received per person when dosed by weight.



The data used in the construction of the curves in Figure 6.7 can be used to choose thresholds between one and two tablets (1v2), two and three tablets (2v3), and three and four tablets (3v4), for dose assignment by height. The process is more easily described and visualised by a graphical than by a tabular explanation. First, an appropriate trade-off between underdosing and overdosing (denoted here by  $\psi$ , where  $\psi$ =number overdosed / number underdosed) must be defined. Then, on the graph, a perpendicular to the horizontal axis (representing the height threshold between doses) is drawn such that the number of individuals to the right of the perpendicular on the curve in the lower weight-based dosing category (overdosed individuals) is  $\psi$  times the number of people to the left of the perpendicular on the curve in the higher weight-based dosing category (underdosed individuals). A  $\psi$  of 10 was (arbitrarily) set for each of the 1v2, 2v3 and

3v4 thresholds. In practice, because height data were grouped at 1cm intervals, it was not possible to achieve a  $\psi$  of exactly 10 (see Table 6.18, p.150).

For the threshold between zero and one tablet (0v1), the curves may be misleading if used in this way, since the zero tablet curve includes a number of individuals who were given suspension simply because they were physically unable to swallow azithromycin tablets, rather than because their body mass was too low to be given 250mg of drug. Further, arbitrary imposition of a trade-off between overdosing and underdosing at this boundary is probably inappropriate. Because children below the one-tablet height limit can be conveniently and reliably weighed using a spring scale, then allocated a dose of suspension (POS), the most important factor in determining the 0v1 threshold is ensuring that long thin children are not given excessive drug. A higher threshold will tend to be safer. To explore the minimum height requirement for one tablet, data from the 430 non-pregnant treated people over the age of twelve months were re-queried, and those who were eligible (by weight) to receive two or more tablets (n=17) excluded. The dose-per-kilogram, should one 250mg azithromycin tablet be administered, was then determined for each of the remaining individuals. If the lower height bound for one tablet is set at 76cm, 199 people would be given one tablet, with the dose given ranging from 15.2–40.3mg/kg. Only five one tablet recipients would receive more than 30mg/kg azithromycin (30.1, 30.1, 30.1, 31.6 and 40.3mg/kg).

The height-based dosing schedule based on these calculations appears in Table 6.17. Table 6.18 (p.150) compares the number of tablets assigned using the current weight-based schedule with the number of tablets assigned using the schedule in Table 6.17. Table 6.19 (p.150) presents an equivalent comparison, but with dose shown in mg/kg body weight rather than in number of tablets.

Table 6.17: Height-based dosing schedule for azithromycin

Height (cm)	Number of tablets
< 76	0 (weigh with spring scale and give POS)
76 – 102	1
103 – 129	2
130 – 143	3
144 +	4

Table 6.18: Comparison of weight-based and height-based dosing (1). People in each weight-based dosing category (rows) are divided by column to indicate the number of tablets that they would have received using the height-based schedule proposed. Cells shaded green are those containing people for whom the number of tablets assigned by height and weight would be the same. People above this green diagonal would be overdosed by the height-based schedule; those below would be underdosed. People assigned to '0 tabs' by either method would be weighed to receive POS.

No. of tablets determined by weight	No. of individuals planned to receive each dose (classed by no. of tablets), if no. of tablets determined by height					Total
	0 tabs	1 tab	2 tabs	3 tabs	4 tabs	
0 tabs	183	80	0	0	0	263
1 tab	39	667	212	0	2	920
2 tabs	0	15	744	170	0	929
3 tabs	0	0	12	404	295	711
4 tabs	1	0	0	16	1938	1955
Total	223	762	968	590	2235	4778

Table 6.19: Comparison of weight-based and height-based dosing (2). As for Table 6.18, but categories here determined by dose of azithromycin tablets in mg/kg body weight. Cells shaded orange contain people who would receive a 'safe and effective' dose using the height-based schedule (or the same dose as that given by weight); those shaded yellow contain 'underdoses'; those shaded red contain 'overdoses'.

Dose in mg/kg of tablets when no. of tablets determined by weight	No. of individuals planned to receive each dose (classed by mg of tablets/kg body weight), if no. of tablets determined by height				Total
	0 (weigh for POS)	0.01 – 14.99mg/kg	15.00 – 29.99mg/kg	30.00 + mg/kg	
0 (give POS)	183	0	75	5	263
0.01 – 14.99mg/kg	0	81 [a]	0	0	81
15.00 – 29.99mg/kg	40 [b]	18 [c]	4000	376	4434
30.00 + mg/kg	0	0	0	0	0
Total	223	99	4075	381	4778

The choice of 15-30mg/kg as the 'safe and effective' dose range is somewhat arbitrary [507], but parallels the range of actual doses received by most individuals dispensed azithromycin tablets by body weight. In Kahe, for example, there were eighty-one individuals (1.7% of 4778) for whom the standard weight-based schedule would deliver less than 15mg azithromycin per kilogram recorded body weight (Table 6.19, cell [a]). All eighty-one were adults due to receive the maximum dose of 1g (four tablets). During the 2000 distribution, in fact, all eighty-one did take four tablets. The height-based schedule is similarly restricted to a four tablet maximum, and correctly assigns four tablets to each of these individuals. Cell [a] has therefore been shaded orange, to indicate a presumed 'safe and effective' dose by height, even though the actual dose received by this group would range from 14.99mg/kg down to 10.91mg/kg.

The height-based schedule refers for a weight-determined POS dose forty individuals (Table 6.19, cell [b]) who would have received a 'safe and effective' 15–30mg/kg of tablets with the weight-based schedule. One of these forty was a twenty year-old woman whose height was (almost certainly erroneously) recorded as 48cm and whose weight was recorded as 39.9kg. Of the other thirty-nine, twenty-nine were unable to swallow an offered tablet, and required suspension in any case. None of the forty would be misdosed as a result of the height-based scheme.

In all, only eighteen people (Table 6.19, cell [c]; 0.4% of 4778) would be underdosed using the height-based schedule, if underdosing is defined as a dose below 15mg/kg body weight *and* less than that offered by the weight-based schedule. All eighteen would be given more than 13mg/kg. Some 4379 people (91.6% of 4778) would receive a presumed safe and effective dose. A total of 381 (8.0% of 4778) would receive more than 30mg/kg. A breakdown of doses determined for this group is presented in Table 6.20. The characteristics of the five individuals scheduled to be given more than 40mg/kg are shown in Table 6.21.

Table 6.20: Dose ranges of those receiving more than 30mg azithromycin per kilogram body weight using the height-based dosing schedule

Dose in mg/kg	Number of individuals in Kahe who would have received this dose, if no. of tablets determined by height (% of 4778 azithromycin-treated people)
30.00 – 32.49	245 (5.1)
32.50 – 34.99	93 (1.9)
35.00 – 37.49	30 (0.6)
37.50 – 39.99	8 (0.1)
40.00+	5 (0.1)

Table 6.21: Further details of the five individuals who would receive more than 40mg azithromycin per kilogram if doses were determined by recorded height. BMI=body mass index.

Weight (kg)	Height (cm)	BMI (kg/m <sup>2</sup> )	Dose by height (no. of tablets)	Dose by schedule (mg/kg)	Actual dose dispensed (no. of tablets)	Actual dose dispensed (mg/kg)
6.2	84	8.8	1	40.3	0 (3mL POS)	19.3
12.0	103	11.3	2	41.7	1	20.8
11.3	113	8.8	2	44.2	1	22.1
15.2	163	5.7	4	65.8	4	65.8
13.6	148	6.2	4	73.5	3	55.1



As shown in Table 6.21, all five individuals slated to receive over 40mg/kg by the height-based dosing schedule were exceptionally tall for their weight. It is not known whether measurement or recording of height or weight were in error.

## 6.5 LONGITUDINAL STUDY: FOLLOW-UP

### 6.5.1 Follow-up success

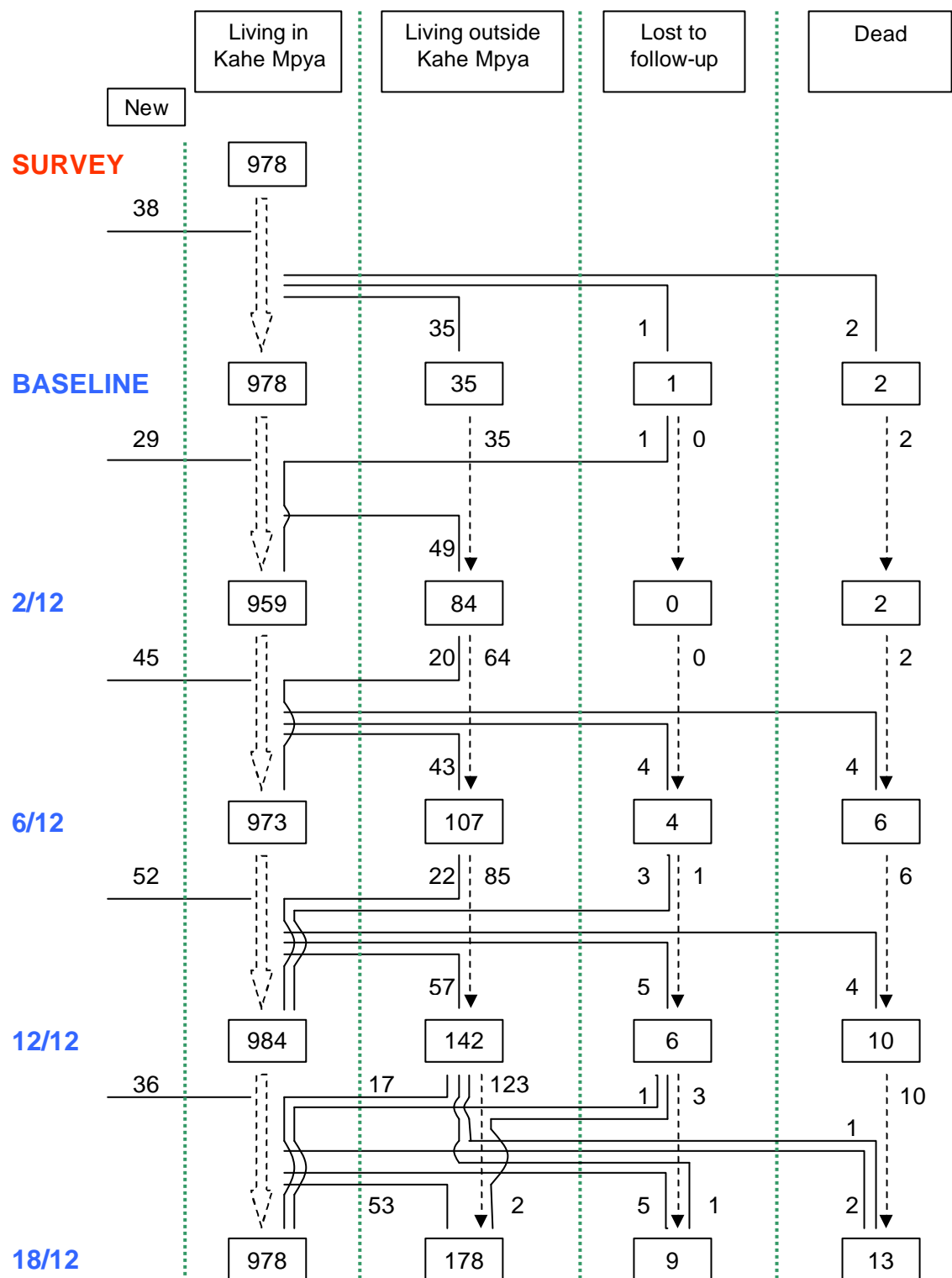
The number of people resident in Kahe Mpya and the number of people examined at each time point are shown in Table 6.22. In this table, and subsequently, the two month follow-up point has been designated ‘2/12’, the six month follow-up ‘6/12’, and so on. Examination coverage at baseline and the four follow-up time points has been calculated as the number of people seen divided by the number of people resident in the sub-village at that time, as determined at the census review conducted immediately prior to examination and swabbing. On this basis, the mean examination coverage for the five rounds was 93%.

The trial profile for the cohort is shown in Figure 6.8 (p.153). Each row in this Figure represents a examination point in either the survey or longitudinal study. At each of those time points, the numbers in boxes show the status (indicated by the column heads) of all individuals registered at any time prior to that point as living in Kahe Mpya. Arrows with solid tails indicate movement of cohort members from one category (e.g., living in Kahe Mpya) to another (e.g., bst to follow-up). Arrows with dashed tails represent individuals remaining in one category between successive examination points. New individuals are introduced from the far left of the Figure. In the interval between the original census and the completion of the final follow-up round eighteen months after treatment, a total of 206 new individuals were born into (n=65) or moved into (n=141) Kahe Mpya.

Table 6.22: Number of people resident in Kahe Mpya and number of people seen at each time point of the longitudinal study

Time point	Number resident	Number seen (% of residents)
Baseline	978	956 (98%)
2/12	959	905 (94%)
6/12	973	879 (90%)
12/12	984	907 (92%)
18/12	978	889 (91%)
Total	4872 (mean=974)	4536 (93%)

Figure 6.8: Longitudinal study profile

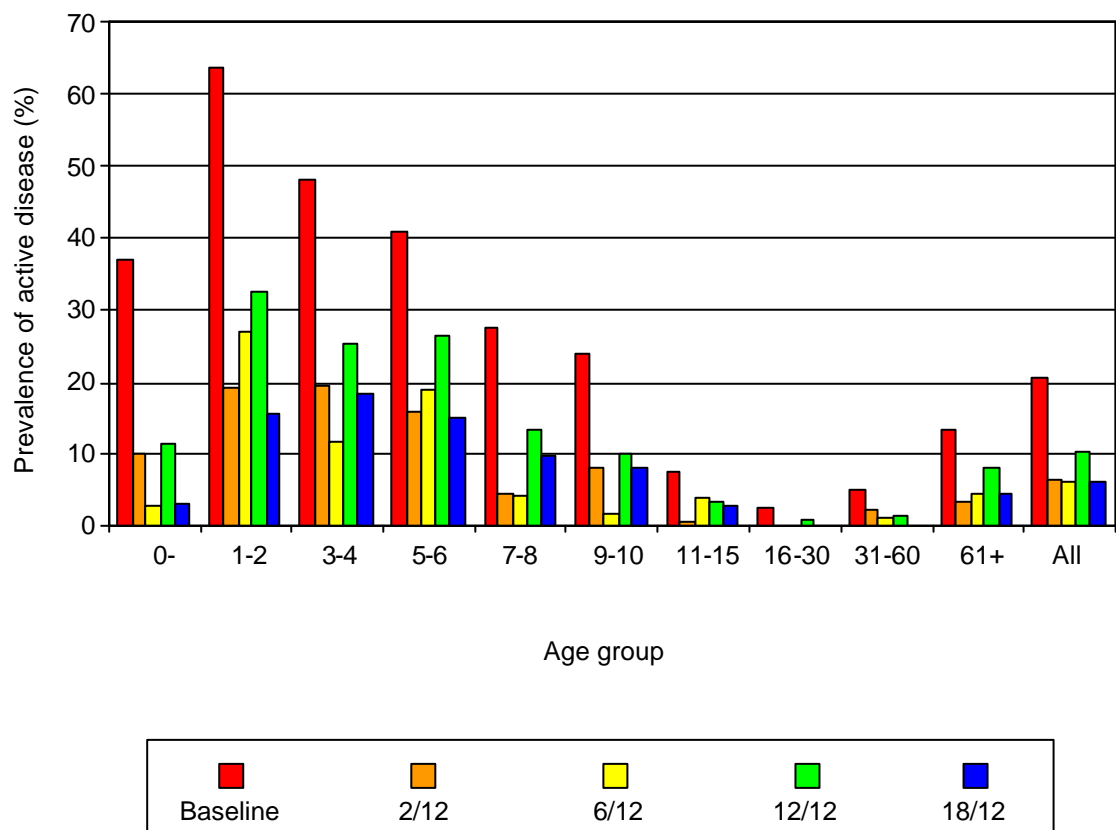


### 6.5.2 Prevalence of active trachoma over time

Figure 6.9 (p.154) shows the prevalence of active trachoma in one or both eyes, by age group, at baseline and the four follow-up points of the longitudinal study. At each time point, the peak in disease prevalence was in young children. The all-ages

prevalence of active disease was significantly lower at each follow-up point than it was at baseline (two-sided  $P < 0.001$  for each comparison). The prevalence at twelve months (94/907, 10.4%) was significantly higher than it was at either six months (54/879, 6.1%) ( $z = 3.15$ ,  $P = 0.0016$ ) or eighteen months (54/889, 6.1%) ( $z = -3.22$ ,  $P = 0.0013$ ). The reason for this finding is unclear. Seasonal fluctuation in transmission of *C. trachomatis* or other ocular pathogens might be responsible: as was suggested in Section 2.6.12(ii) (p.63), it has been hypothesised that a variety of micro-organisms might be able to induce conjunctival changes reminiscent of active trachoma in individuals who have previously been exposed to ocular *Chlamydia*. The prevalence of TF in one to nine year-old children was 117/325 (36.0%) at baseline, 41/311 (13.2%) at two months, 36/295 (12.2%) at six months, 66/319 (20.7%) at twelve months, and 39/315 (12.4%) at eighteen months: a profile of prevalence over time similar to that displayed for all-ages active disease in Figure 6.9.

Figure 6.9: Prevalence of active disease by age group over time



### 6.5.3 Prevalence of infection over time

The observed prevalence of *C. trachomatis* positive swabs at each time point is shown in Table 6.23. The prevalence of conjunctival infection progressively decreased over the course of follow-up. The decrease from baseline to two months was statistically significant ( $z=-6.69$ ,  $P<0.0001$ ). At six months, twelve months and eighteen months, the prevalence of ocular positivity remained significantly lower than it was before treatment ( $P<0.0001$  for each comparison), but was not significantly below that recorded for the previous round.

The proportion of nasal exudate swabs that were *C. trachomatis* positive progressively decreased from baseline up to twelve months, then increased from 1% to 3%. At each follow-up point (two, six, twelve and eighteen months), the proportion positive was significantly lower than that at baseline. The increase in the prevalence of positivity from twelve to eighteen months was not significant ( $z = 0.67$ ,  $P = 0.50$ ).

Table 6.23: Amplicor results for primary eye swabs, duplicate eye swabs and swabs of nasal exudate against time

Time point	No. seen	Number Amplicor positive/number taken (%)		
		Primary eye swabs	Duplicate eye swabs	Nasal exudate swabs
Baseline	956	91/956 (10%)	5/43 (12%)	19/67 (28%)
2/12	905	19/905 (2%)	not done	6/53 (11%)
6/12	879	13/879 (1%)	not done	2/30 (7%)
12/12	907	8/907 (1%)	not done	1/79 (1%)
18/12	889	5/889 (1%)	not done	1/32 (3%)
Total	4536	136/4536 (3%)	5/43 (12%)	29/261 (11%)

### 6.5.4 Intensity of ocular infection over time

Figures 6.10 through 6.13 (p.156–157) are scatter plots of the estimated number of copies of *omp1* per swab by age and gender at two, six, twelve and eighteen months respectively. As for the baseline data plotted in Figure 6.4 (p.142), only data points relating to samples positive by Amplicor (copies *omp1*>0) are included. At two months, the highest *omp1* swab load was 4264 copies *omp1* (compared to 491708 at baseline); at six months, it was 2164; at twelve months, it was 236; and at eighteen months, it was 14178. In general, as at baseline, higher loads were found in younger individuals, though a sixty-two year-old woman and an eighty-eight year-old woman both had low-level positive swabs at eighteen months (Figure 6.13, p.157). There were no other CT-positive individuals at eighteen months in the household (or even balozi) in which either woman was living.

Figure 6.10: Copies *omp1* per swab by age and gender at two months for subjects with an Amplicor positive swab ('o'=male, '+'=female)

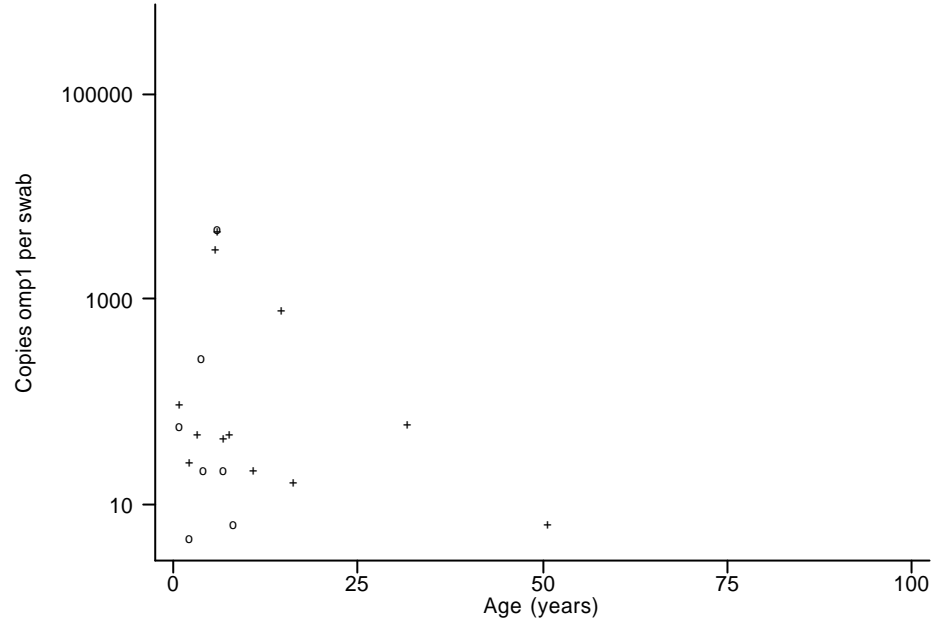


Figure 6.11: Copies *omp1* per swab by age and gender at six months for subjects with an Amplicor positive swab ('o'=male, '+'=female)

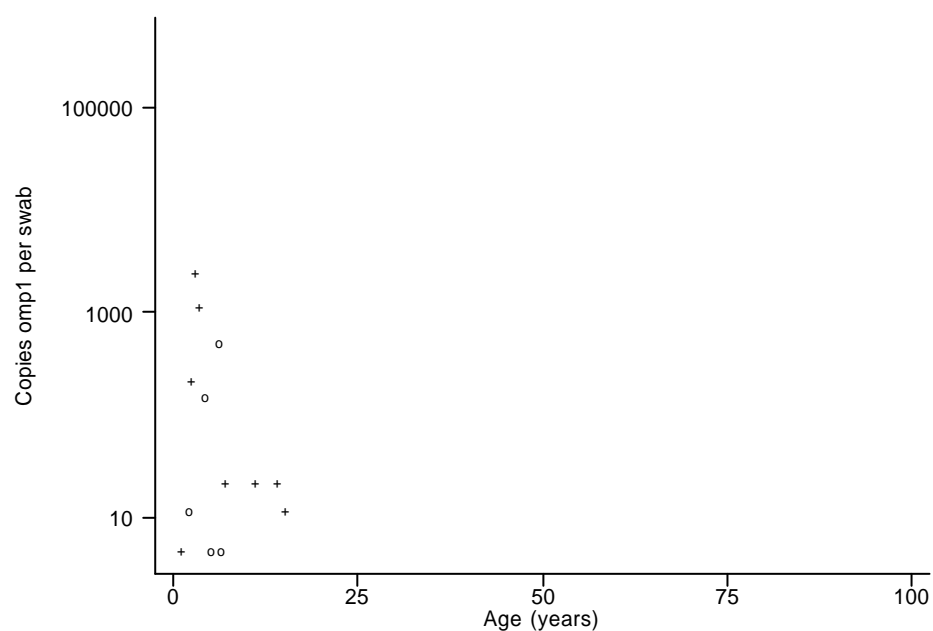


Figure 6.12: Copies *omp1* per swab by age and gender at twelve months for subjects with an Amplicor positive swab ('o'=male, '+'=female)

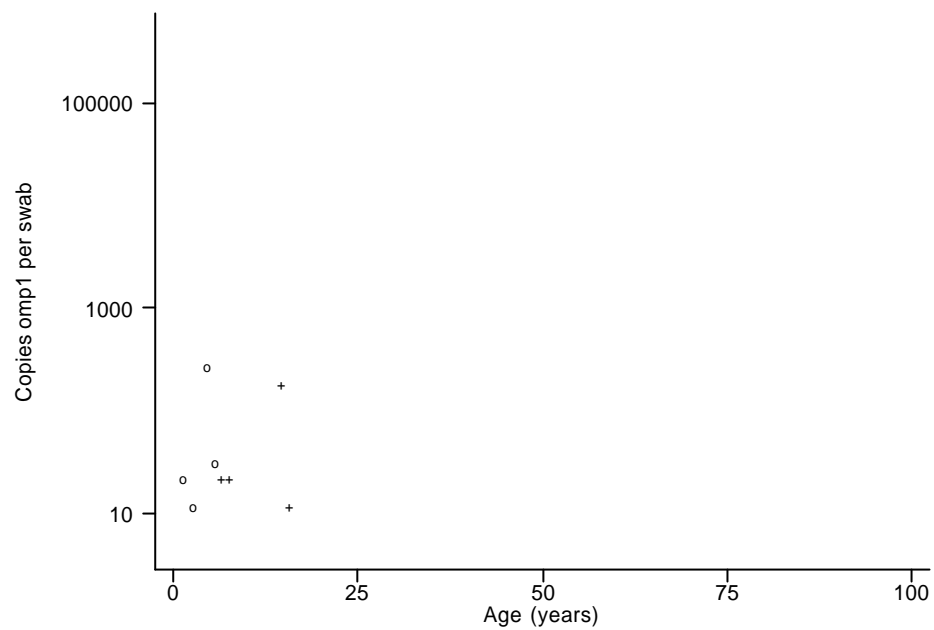
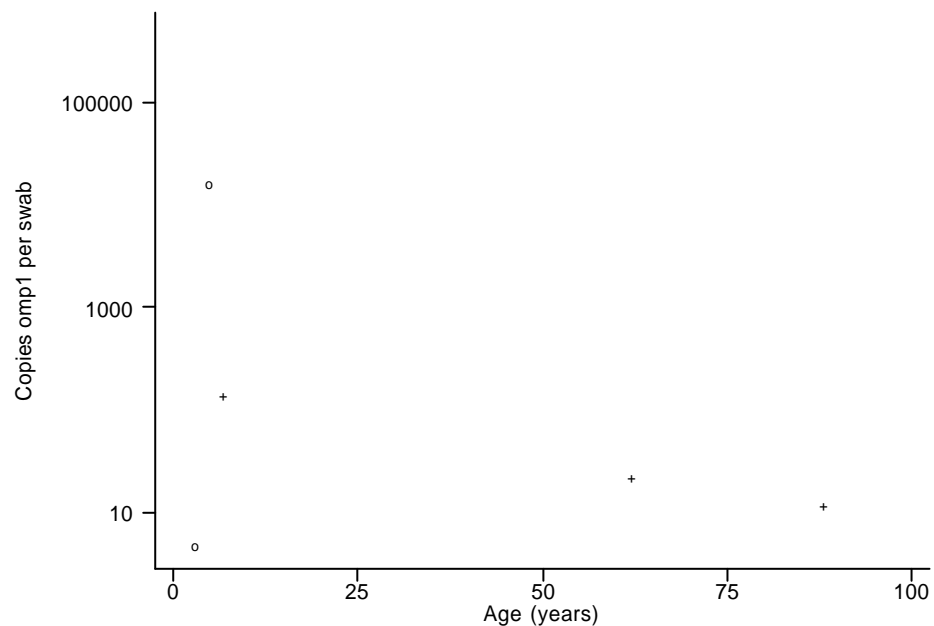


Figure 6.13: Copies *omp1* per swab by age and gender at eighteen months for subjects with an Amplicor positive swab ('o'=male, '+'=female)



Frequency distributions of the number of copies of *omp1* per swab found amongst those with Amplicor positive swabs at each time point comprise Figure 6.14 through Figure 6.18 (p.158–159). Table 6.24 (p.159) shows the COCTL for the cohort at each time point; these data, including the 95% confidence intervals, are shown in Figure 6.19 (p.160). The COCTL is shown by age group in Figure 6.20 (p.160), and by age group and gender in Figure 6.21 (p.160).

Figure 6.14: Frequency distribution of copies *omp1* per swab in Amplicor positive subjects at baseline

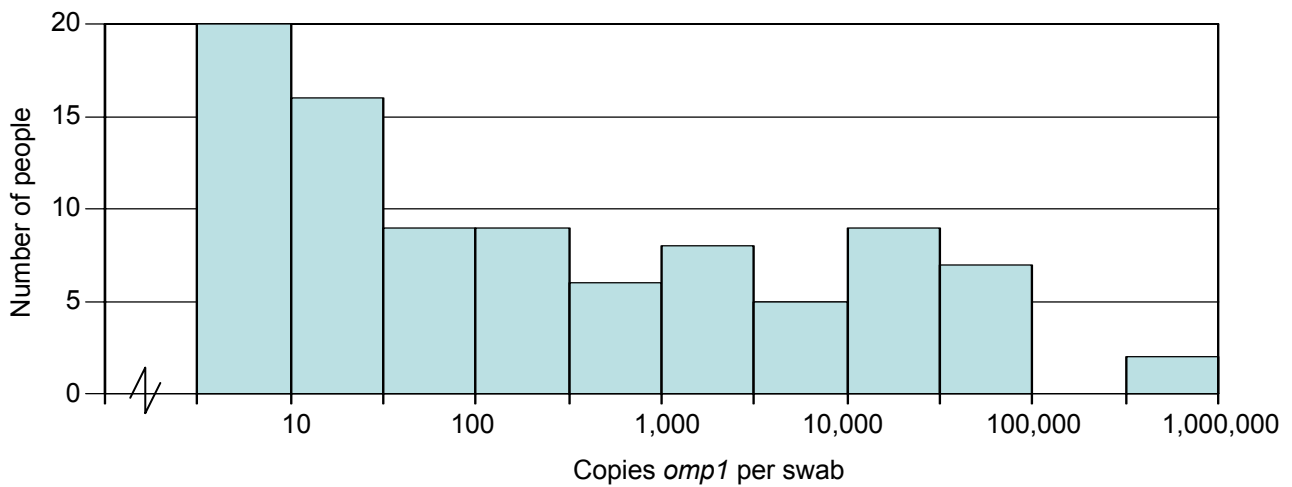


Figure 6.15: Frequency distribution of copies *omp1* per swab in Amplicor positive subjects at two months

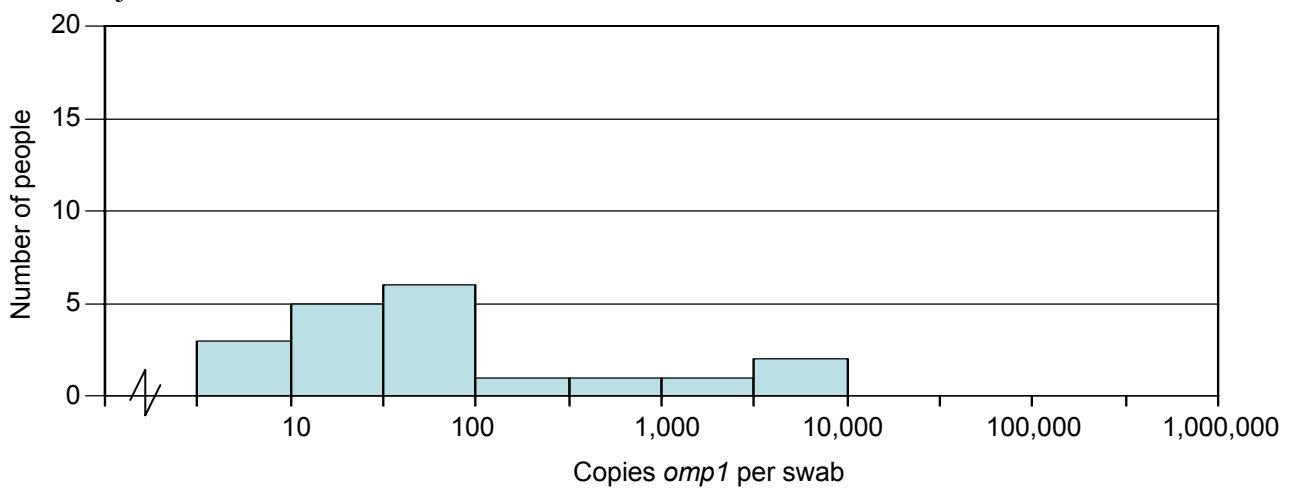


Figure 6.16: Frequency distribution of copies *omp1* per swab in Amplicor positive subjects at six months

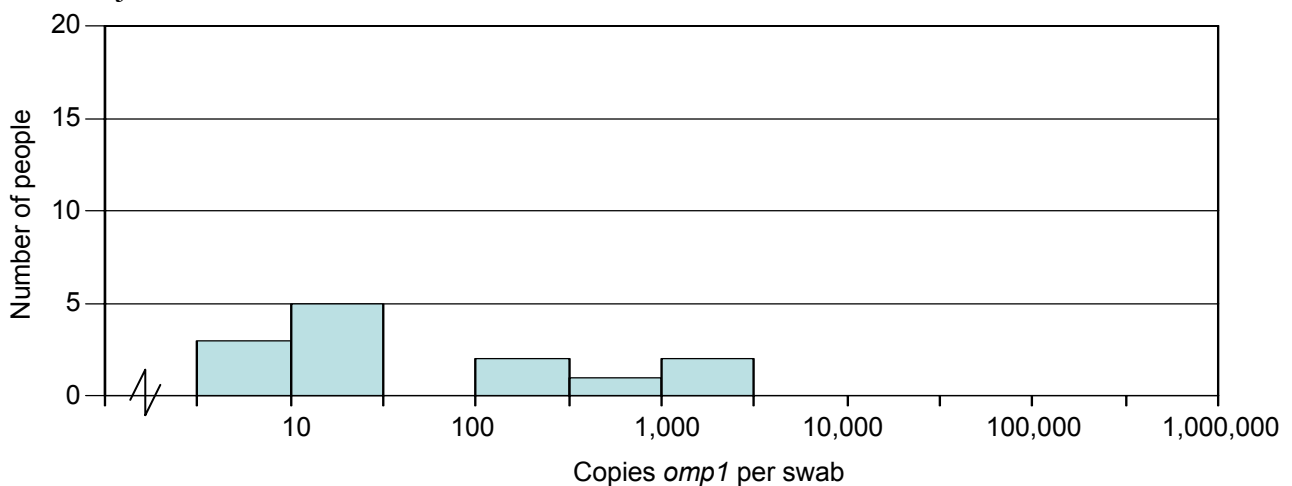


Figure 6.17: Frequency distribution of copies *omp1* per swab in Amplicor positive subjects at twelve months

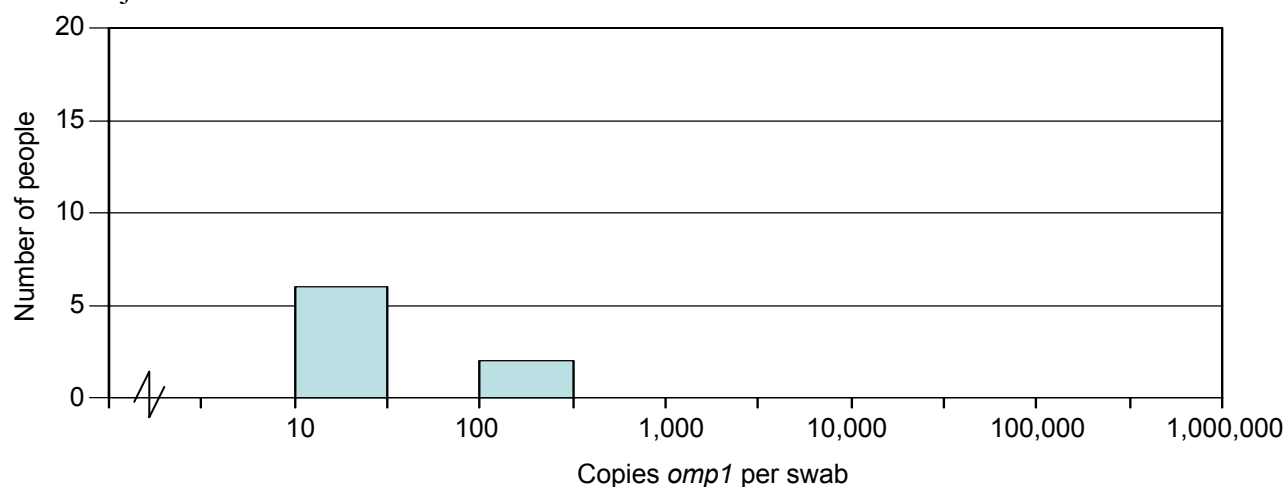


Figure 6.18: Frequency distribution of copies *omp1* per swab in Amplicor positive subjects at eighteen months

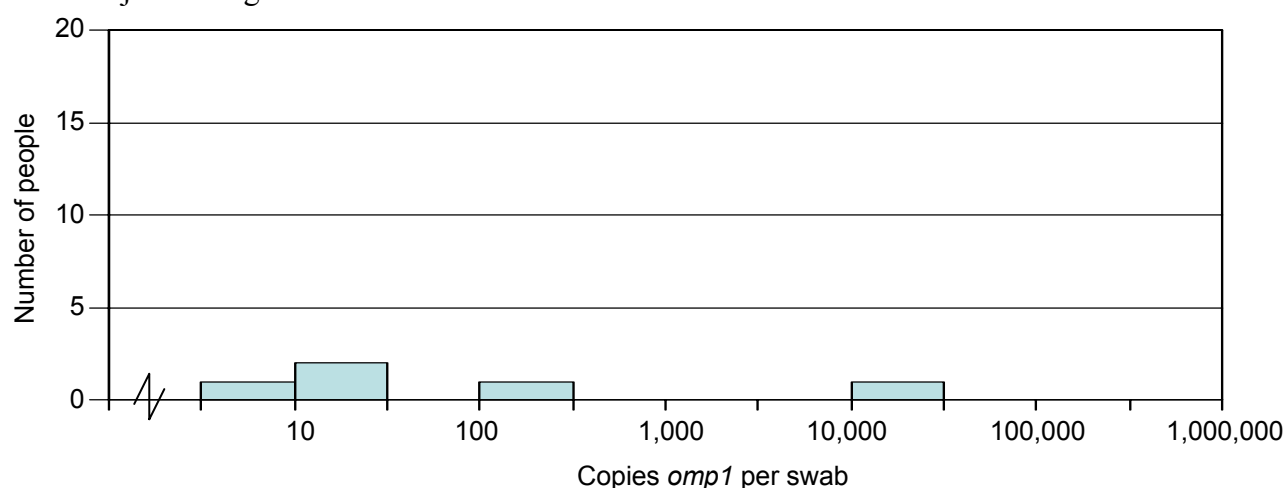


Table 6.24: COCTL, all ages and both genders, at each time point. Confidence intervals determined by bootstrapping, with 10,000 replicates.

Time point	No. seen	COCTL (95% CI), [% of baseline]
Baseline	956	1.99 (1.44 - 2.67), [100]
2/12	905	0.26 (0.14 – 0.43), [13]
6/12	879	0.16 (0.07 – 0.29), [8]
12/12	907	0.08 (0.03 – 0.16), [4]
18/12	888	0.07 (0.01 – 0.19), [4]



Figure 6.19: COCTL, all ages and both genders, against time (vertical bars show 95% CI at each time point)

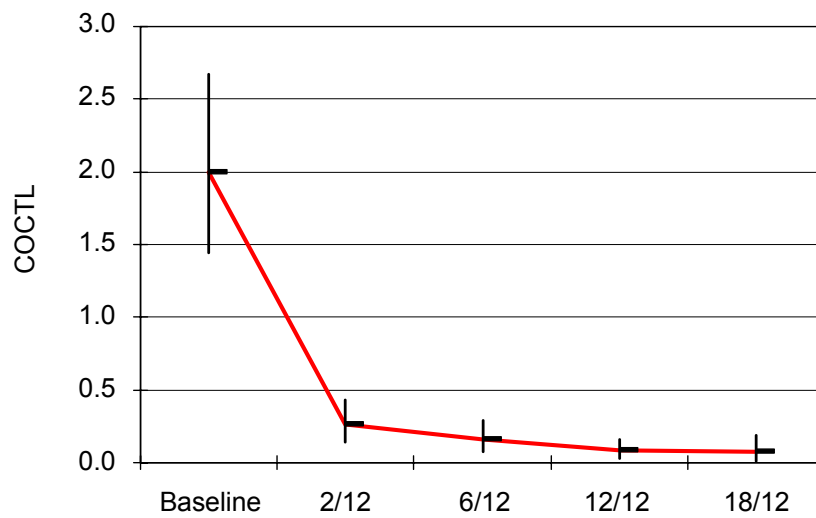


Figure 6.20: COCTL by age group against time

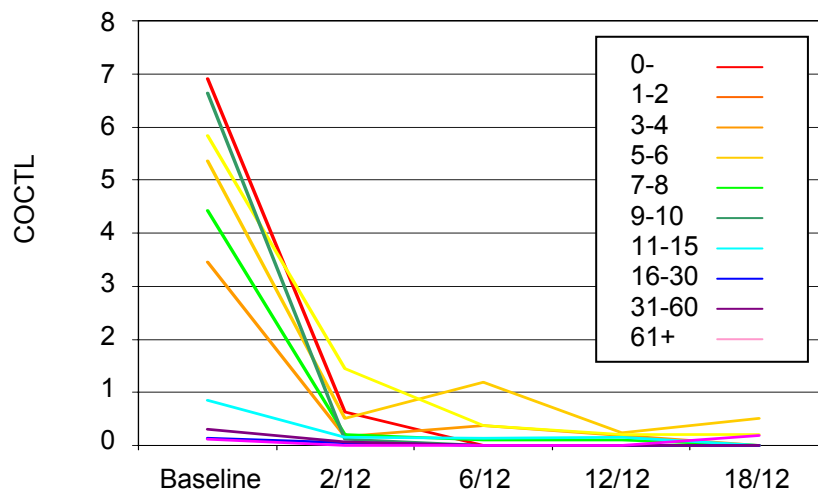
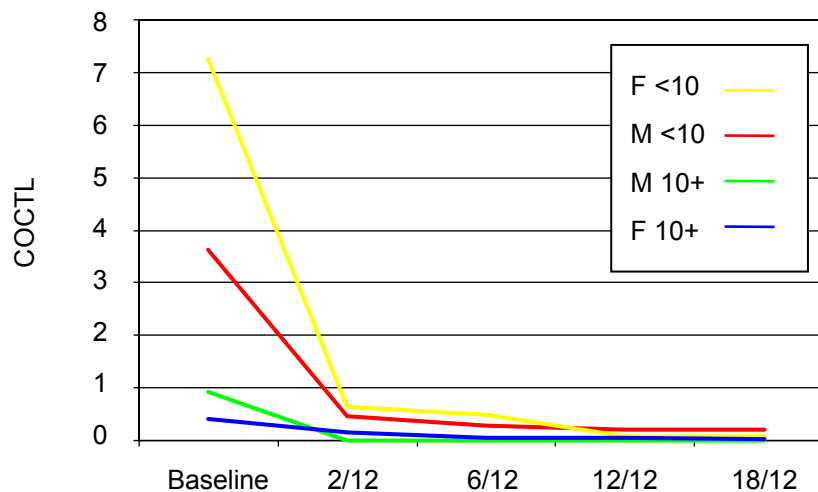


Figure 6.21: COCTL by age group and gender against time



The observed reductions in COCTL following treatment were striking. For the community as a whole (Figure 6.19), the index dropped to 13% of its pre-treatment level two months after mass distribution of single-dose azithromycin. Thereafter, it continued to fall, being 8% of its baseline level at six months, 4% at twelve months, and 4% at eighteen months. Falls in COCTL were observed in all age and gender groups (Figure 6.20 and Figure 6.21). Other than the supply of two tubes of tetracycline eye ointment to individuals with clinical evidence of active disease at the six month (fifty-four people) and twelve month (ninety-four people) follow-up points, there were no other formal trachoma control efforts undertaken in the village during this period. The possible contribution of topical treatment to the observed reduction in infection intensity will be discussed in Section 7.3.4 (p.187).

In Table 6.25, the ages for the 50<sup>th</sup> and 90<sup>th</sup> centiles of the cumulative load of ocular *C. trachomatis* are shown for each time point. These are the ages below which 50% and 90% respectively of the total ocular chlamydial burden was found. The 50<sup>th</sup> centile was consistently located below six years of age; the 90<sup>th</sup> centile was consistently below fifteen years of age. These data underline the importance of children as a reservoir of ocular CT.

Table 6.25: Cumulative ocular *C. trachomatis* load with increasing age at each time point

Time point	Age at 50 <sup>th</sup> centile	Age at 90 <sup>th</sup> centile
Baseline	0 years 9 months	6 years 8 months
2/12	5 years 11 months	6 years 0 months
6/12	3 years 1 month	6 years 2 months
12/12	4 years 7 months	14 years 7 months
18/12	5 years 0 months	5 years 0 months

### 6.5.5 Risk factors for ocular infection at two months

A description of and rationale for the methods used to investigate risk factors for post-treatment infection were both presented in Section 5.9.3 (p.125). Everyone swabbed at two months who had received tetracycline eye ointment at baseline or one month lived in a house with access to a latrine. This meant that baseline tetracycline use and latrine access were collinear in the regression model. When latrine access was dropped from the first phase of analysis, tetracycline use was not significantly associated with the risk of having a positive ocular swab, but when tetracycline use was dropped, latrine access was associated with decreased risk of being Amplicor positive.

Individuals with ocular or nasal discharge were significantly more likely than others to be positive. These results are shown in Table 6.26.

Table 6.26: Logistic regression results for risk of being Amplicor positive at two months. Information about latrine access was not available for 29 individuals. Only exposures for which the adjusted odds ratios were statistically significant are shown.

‘Exposure’	Prevalence of ‘exposure’ exposed/seen (%)	Crude (unadjusted) odds ratio	Adjusted odds ratio (95% CI)
Access to a latrine	870/876 (99.3)	0.037	0.029 (0.0048–0.17)
Nose discharge	53/905 (5.9)	4.6	4.7 (1.4–15)
Eye discharge	2/905 (0.2)	49	32 (3.8–263)

#### 6.5.6 Risk factors for ocular infection at six months

None of the individuals examined at this time point had any ocular discharge visible on examination, so the ‘exposure’ ‘eye discharge’ was dropped from the analysis. Only two factors were significantly associated with the risk of being Amplicor positive at six months: increasing age, which was associated with reduced risk of being positive, and the presence of a visible nasal discharge, which was associated with an increased risk (Table 6.27). The odds ratios shown for age are for each additional year of age, suggesting a progressively reduced risk of being ocular CT positive in older people.

Table 6.27: Logistic regression results for risk of being Amplicor positive at six months. Only exposures for which the adjusted odds ratios were statistically significant are shown.

‘Exposure’	Prevalence of ‘exposure’ exposed/seen (%)	Crude (unadjusted) odds ratio	Adjusted odds ratio (95% CI)
Age (for each year)	not applicable	0.89	0.91 (0.86–0.97)
Nose discharge	30/879 (3.4)	14	6.7 (2.0–22.4)

#### 6.5.7 Risk factors for ocular infection at twelve months

At twelve months, none of the seventy-nine individuals with nasal discharge, the nineteen individuals with examiner-observed fly-eye contact, or the eight individuals with ocular discharge had swabs that tested positive by Amplicor in the laboratory. These three ‘exposures’ were therefore eliminated from the analysis. All eight twelve month Amplicor positive individuals had access to a latrine. This ‘exposure’ was therefore also not considered. Only two terms returned significant odds ratios in the

regression model (Table 6.28). As was noted at six months, at twelve months, increasing age was associated with decreasing risk of having an Amplicor positive conjunctival swab. Receipt of tetracycline eye ointment at the six month examination was associated with a significantly increased risk of being Amplicor positive at twelve months.

Table 6.28: Logistic regression results for risk of being Amplicor positive at twelve months. Only exposures for which the adjusted odds ratios were statistically significant are shown.

‘Exposure’	Prevalence of ‘exposure’ exposed/seen (%)	Crude (unadjusted) odds ratio	Adjusted odds ratio (95% CI)
Age (for each year)	not applicable	0.91	0.94 (0.89–0.99)
Tetracycline at 6/12	52/907 (5.7)	18	11 (2.8–45)

#### 6.5.8 Risk factors for ocular infection at eighteen months

All five Amplicor positive individuals at eighteen months had been treated with azithromycin at baseline or one month; none, therefore, had been given tetracycline at either time. None of the five Amplicor positive subjects had either ocular or nasal discharge on examination. The exposure variables representing treatment at baseline or one month, and eye and nose discharge at twelve months were therefore dropped. According to the model, when the effect of other variables is controlled, increasing age was associated with an *increased* risk of infection at eighteen months (Table 6.29), in contrast to the reduction in risk with increasing age seen in the analyses for the previous two time points (see Sections 6.5.6 and 6.5.7 above). As at twelve months, at eighteen months, people who had received tetracycline eye ointment at the previous examination were significantly more likely than others to have an Amplicor positive eye swab. Access to a latrine was associated with decreased risk of having a positive swab.

Table 6.29: Logistic regression results for risk of being Amplicor positive at eighteen months. Only exposures for which the adjusted odds ratios were statistically significant are shown.

‘Exposure’	Prevalence of ‘exposure’ exposed/seen (%)	Crude (unadjusted) odds ratio	Adjusted odds ratio (95% CI)
Age (for each year)	not applicable	1.02	1.04 (1.004–1.1)
Tetracycline at 12/12	88/889 (10)	14	18 (3.2–100)
Access to a latrine	873/889 (98)	0.22	0.014 (0.0020–0.094)

### **6.5.9 Spatial distribution of ocular infection over time**

Figure 6.22 through Figure 6.25 (p.166–169) are maps of the distribution of ocular infection by household at two months, six months, twelve months and eighteen months. On the two month and twelve month maps, the locations of the ‘most likely’ clusterings identified in the SaTScan analysis are shown as circles. At six months and eighteen months, none of the clusterings identified in the analysis were statistically significant. The significant clusterings demonstrated at baseline, two months and twelve months do not overlap. The hypothesis underlying these analyses was that there might be fixed exposures in or near Kahe Mpya predisposing local residents to an elevated risk of ocular CT infection. Based on these data, no support is found for such a hypothesis.

### **6.5.10 Relationship between signs of active disease and prevalence and intensity of ocular infection**

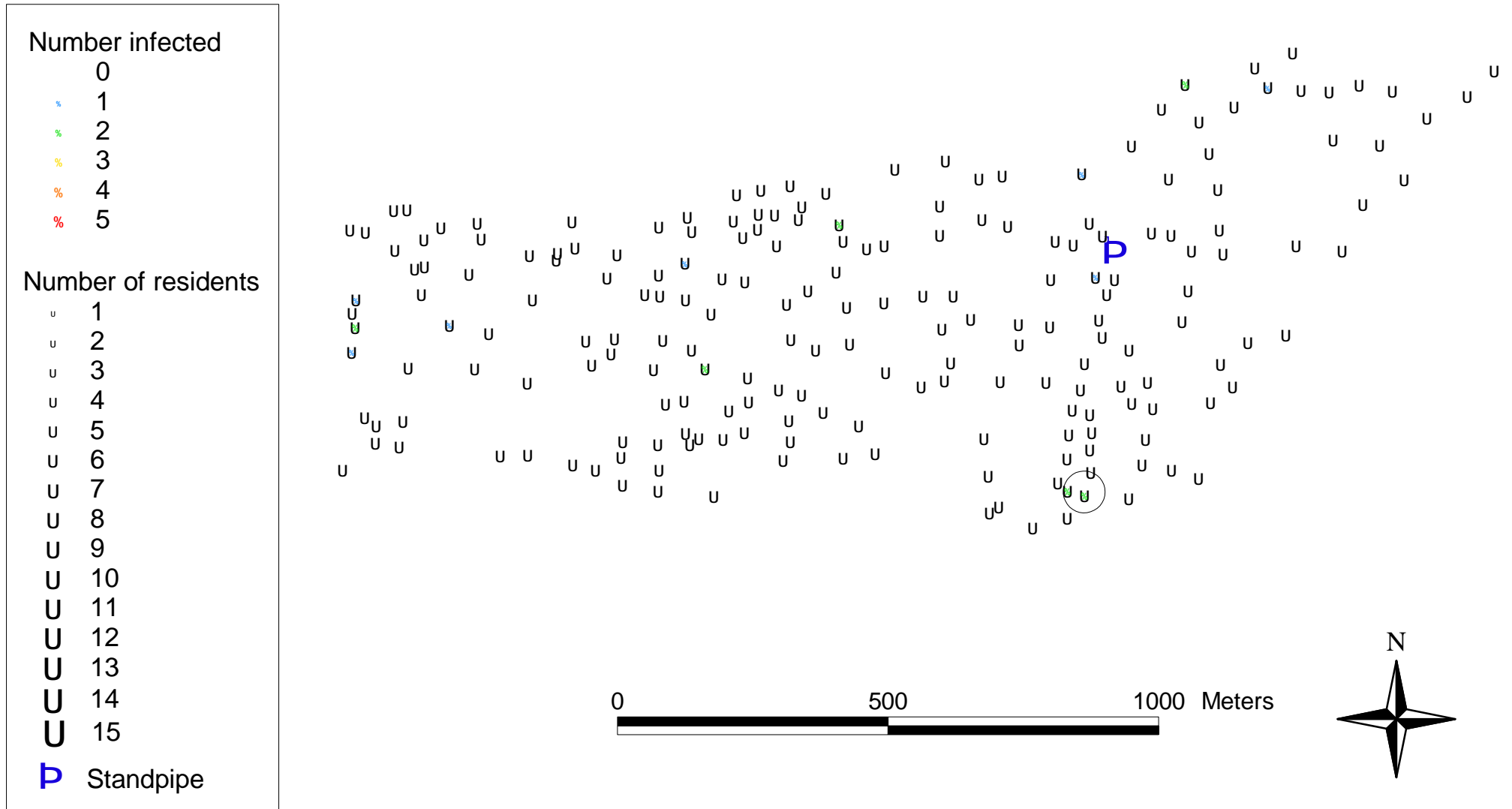
The prevalence of infection in different active disease clinical categories is shown in Table 6.30 (p.170). At baseline, the highest prevalence of Amplicor positivity (55%) was noted in individuals who had both TF and TI, while only 4% of individuals without signs of active disease were Amplicor positive. Similarly, at the two, six and eighteen month follow-up points, individuals with both TF and TI had the highest prevalence of infection, and those without either sign were rarely infected. At twelve months, however, 0/12 individuals with both TF and TI were infected.

There is a similar lack of consistency in relative values of COCTL by sign of active disease, which are tabulated in Table 6.31 (p.170). There was a trend for people with both TF and TI to have the highest ocular CT loads, but this was not the case at the twelve month follow-up point, when none of the individuals in this clinical category were infected.

Table 6.32 (p.171) shows the proportion of the total ocular CT load in the community found in individuals in each active disease clinical category. No clinical category consistently represented more than half of the total ocular CT load. Though individuals without signs of active disease had a very low prevalence of infection (Table 6.30) and a very low COCTL (Table 6.31) at each time point, at both two months and twelve months they harboured more than a quarter of the total community ocular CT, and at six months they had 60%. Considered together, these data make it difficult to support the use of clinical signs for directing community-based antibiotic treatment aimed at the elimination of trachoma. Determining target groups by age, rather than

clinical status, would be more logical in this setting, for two reasons. First, the bulk of the community ocular CT load was consistently found in children, both before azithromycin treatment and at each post-treatment time point (Table 6.25, p.161). Second, the use of age thresholds is simpler and requires less specialised personnel. This will be discussed further in Section 7.4.2 (p.188).

**Figure 6.22: Distribution of Amplicor positive individuals (by household) at two months**



**Figure 6.23: Distribution of Amplicor positive individuals (by household) at six months**

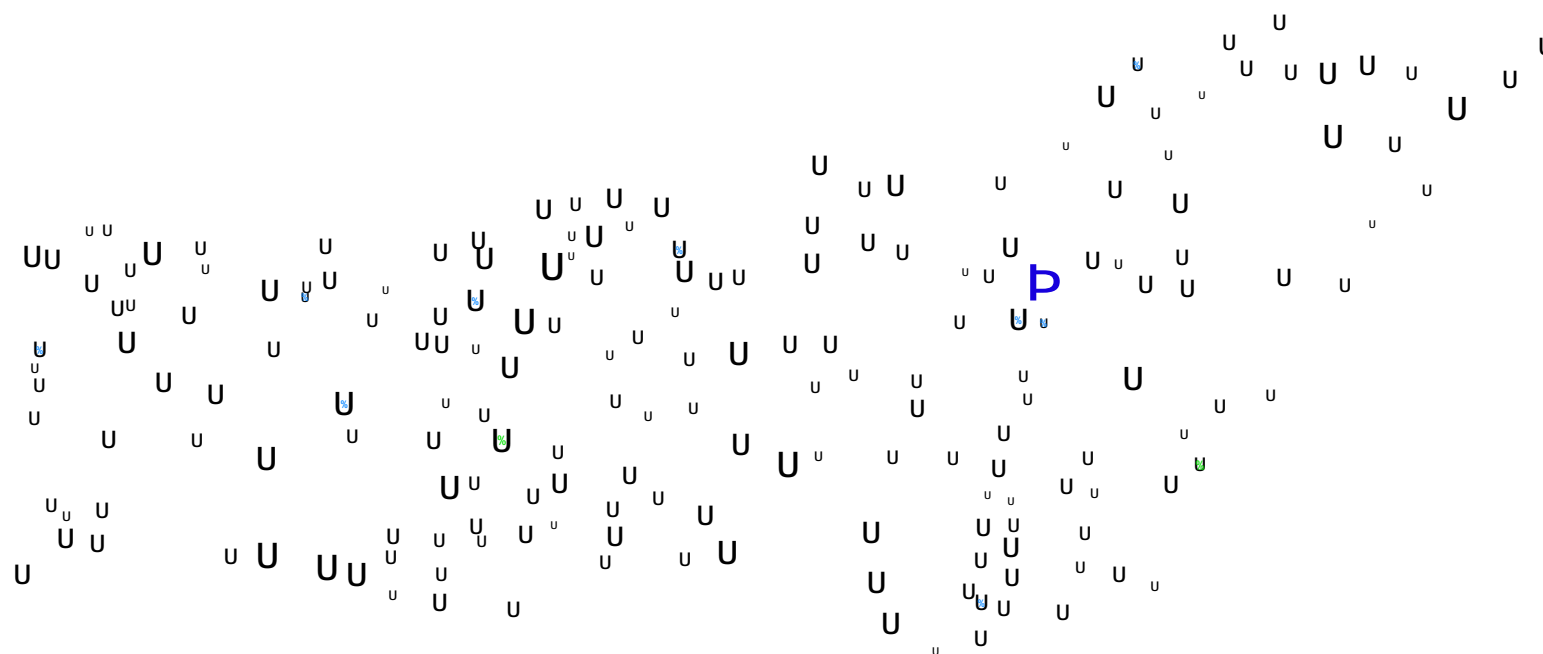
Number infected

- 0
- % 1
- % 2
- % 3
- % 4
- % 5

Number of residents

- u 1
- u 2
- u 3
- u 4
- u 5
- u 6
- u 7
- u 8
- u 9
- u 10
- u 11
- u 12
- u 13
- u 14
- u 15

**P** Standpipe





**Figure 6.24: Distribution of Amplicor positive individuals (by household) at twelve months**

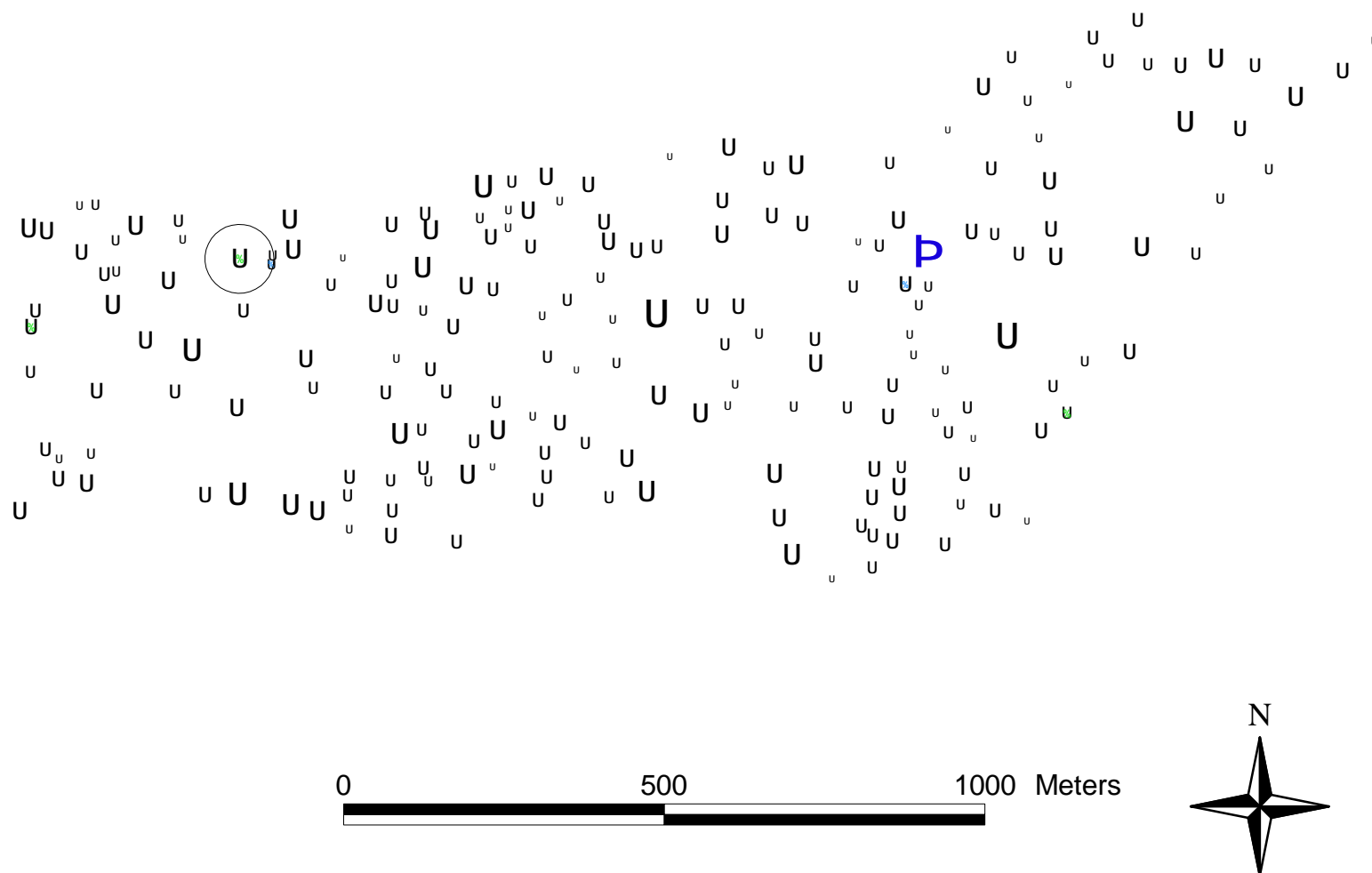
Number infected

0  
 % 1  
 % 2  
 % 3  
 % 4  
 % 5

Number of residents

1  
 2  
 3  
 4  
 5  
 6  
 7  
 8  
 9  
 10  
 11  
 12  
 13  
 14  
 15

**P** Standpipe



**Figure 6.25: Distribution of Amplicor positive individuals (by household) at eighteen months**

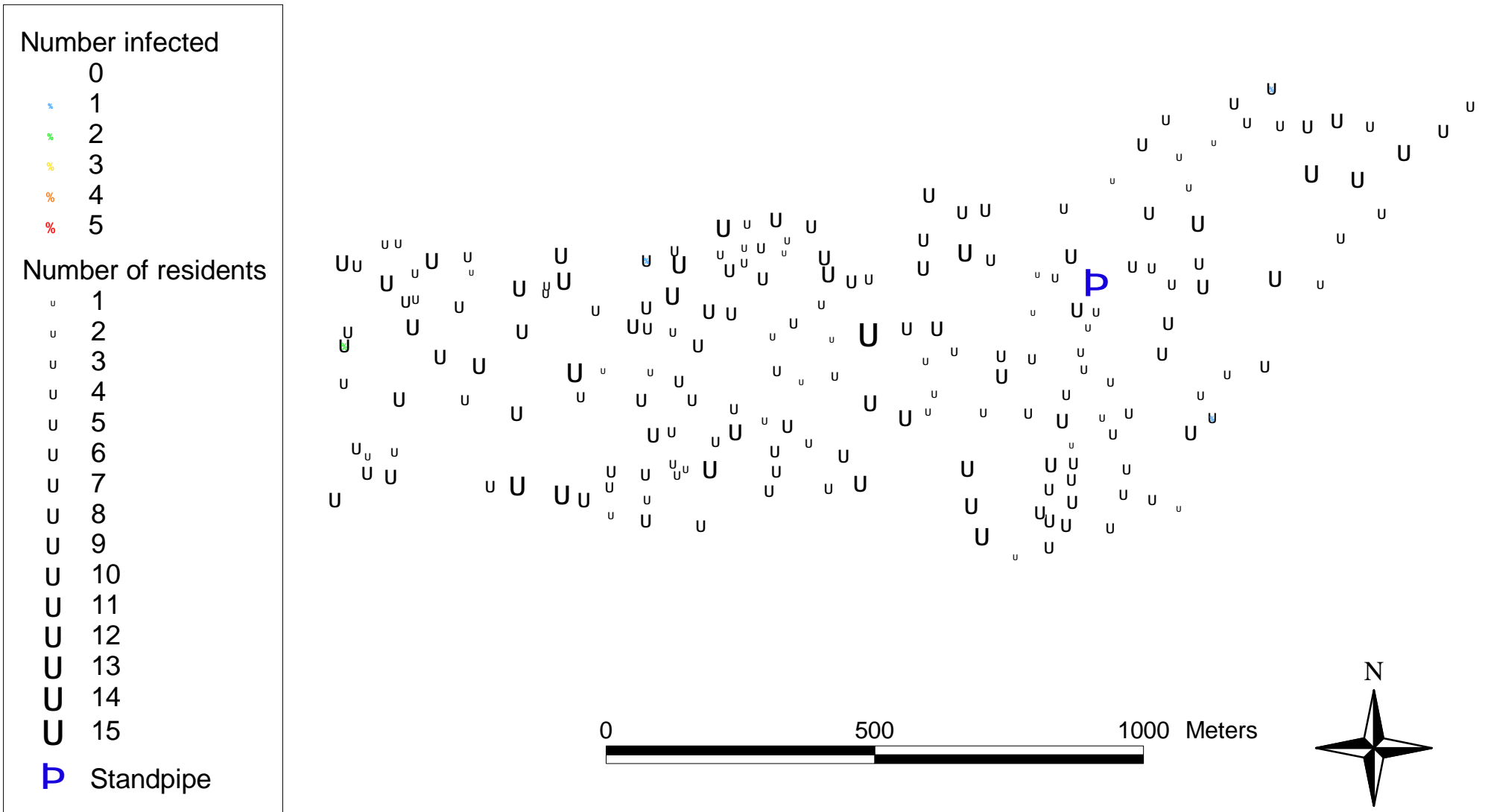


Table 6.30: Prevalence of infection by signs of active disease in the swabbed eye, at each time point. The sum of the *ns* shown in each column is greater than the number of people seen at that time point because the clinical categories used are not mutually exclusive.

Clinical status (right eye)	Number positive by Amplicor/number in that clinical category (%)				
	Baseline	2/12 <sup>xxx</sup>	6/12	12/12	18/12
TF but not TI	17/63 (27)	4/25 (16)	3/17 (18)	5/47 (11)	1/21 (5)
TI but not TF	11/56 (20)	1/9 (11)	0/14 (0)	0/18 (0)	0/11 (0)
Both TF and TI	30/55 (55)	4/15 (27)	3/11 (27)	0/12 (0)	2/11 (18)
TF (regardless of TI)	47/118 (40)	8/40 (20)	6/28 (21)	5/59 (8)	3/32 (9)
TI (regardless of TF)	41/111 (37)	5/24 (21)	3/25 (12)	0/30 (0)	2/22 (9)
TF and/or TI	58/174 (33)	9/49 (18)	6/42 (14)	5/77 (6)	3/43 (7)
Neither TF nor TI	33/782 (4)	10/855 (1)	7/837 (1)	3/830 (0)	2/845 (0)

Table 6.31: COCTL by signs of active disease in the swabbed eye, at each time point. Confidence intervals determined by bootstrapping, with 10,000 replicates. The sum of the *ns* shown in each column is greater than the number of people seen at that time point because the clinical categories used are not mutually exclusive.

Clinical status (right eye)	COCTL (95% CI), <i>n</i>				
	Baseline	2/12 <sup>xxx</sup>	6/12	12/12	18/12
TF but not TI	7.8 (3.1 – 18), 63	3.5 (0.4 – 13), 25	2.0 (0.2 – 9.3), 17	1.1 (0.3 – 2.7), 47	0.7 (0.0 – 5.2), 21
TI but not TF	8.9 (2.7 – 26), 56	1.7 (0.0 – 19), 9	0.0 (0.0 – 0.0), 14	0.0 (0.0 – 0.0), 18	0.0 (0.0 – 0.0), 11
Both TF and TI	111 (41 – 300), 55	5.5 (1.1 – 30), 15	5.8 (0.3 – 34), 11	0.0 (0.0 – 0.0), 12	5.1 (0.0 – 81), 11
TF (regardless of TI)	30 (16 – 58), 118	4.2 (1.2 – 12), 40	3.3 (0.7 – 9.9), 28	0.8 (0.2 – 2.0), 59	1.9 (0.1 – 8.8), 32
TI (regardless of TF)	34 (17 – 72), 111	3.9 (0.8 – 13), 24	2.0 (0.1 – 8.0), 25	0.0 (0.0 – 0.0), 30	2.0 (0.0 – 15.2), 22
TF and/or TI	21 (12 – 37), 174	3.7 (1.3 – 9.4), 49	2.0 (0.4 – 5.5), 42	0.6 (0.2 – 1.5), 77	1.4 (0.1 – 5.9), 43
Neither TF nor TI	0.3 (0.2 – 0.5), 782	0.1 (0.0 – 0.2), 855	0.1 (0.0 – 0.2), 837	0.0 (0.0 – 0.1), 830	0.0 (0.0 – 0.0), 845

<sup>xxx</sup> Examination data were missing from one sixty-year-old woman at two months. Her two-month swab was negative, as were the swabs taken from her at baseline and six, twelve and eighteen months. She had bilateral conjunctival scarring, but neither TF nor TI was found at any of the four time points for which clinical details are available. She has not been included in Table 6.30, Table 6.31 or Table 6.32, so the total of those with TF but not TI, TI but not TF, both TF and TI, and neither TF nor TI shown in these Tables adds to 904 rather than 905.

Table 6.32: Contribution to the total community ocular *C. trachomatis* load by subjects with different categories of active disease in the swabbed eye, at each time point. The number of individuals in each category can be read from Table 6.31. The sum of the contributions from the different clinical categories at each time point is greater than 100%, because the clinical categories used are not mutually exclusive.

Clinical status (right eye)	Total number of copies <i>omp1</i> in swabs from people in that clinical category (% of total community copies <i>omp1</i> )				
	Baseline	2/12	6/12	12/12	18/12
TF but not TI	89235 (6)	4575 (37)	461 (11)	312 (62)	121 (1)
TI but not TF	1034984 (66)	85 (1)	0 (0)	0 (0)	0 (0)
Both TF and TI	424229 (27)	4136 (33)	1157 (29)	0 (0)	14183 (99)
TF (regardless of TI)	513464 (33)	8711 (70)	1618 (40)	312 (62)	14304 (100)
TI (regardless of TF)	1459213 (92)	4221 (34)	1157 (29)	0 (0)	14183 (99)
TF and/or TI	1548447 (98)	8796 (71)	1618 (40)	312 (62)	14304 (100)
Neither TF nor TI	30276 (2)	3640 (29)	2427 (60)	192 (38)	30 (0)
All subjects	1578724 (100)	12436 (100)	4045 (100)	503 (100)	14334 (100)

## 7. DISCUSSION

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### 7.1 GENERAL

The results of this study have major significance for trachoma control programmes. They show – in the setting studied, at least – that (1) the quantitative bulk of ocular *C. trachomatis* is found in young children; (2) a dramatic and sustained reduction in the community ocular CT burden is achievable with mass antibiotic treatment; (3) age is a better measure than clinical signs for determining target groups for antibiotic treatment; and (4) height could safely be used in place of weight to determine the number of azithromycin tablets required for each individual. Before these arguments can be sustained, though, there is a need to establish both the reliability of the data and the logic of the assumptions made as part of their interpretation. In particular, is it reasonable to propose that the quantitative-PCR-estimated concentration of *omp1* in a sample of conjunctival swab extract is related to the load of ocular *C. trachomatis* in the swabbed individual? Is Q-PCR the best assay for determining this load? And what evidence exists to suggest that *C. trachomatis* load has clinical or epidemiological relevance? These three questions are addressed in Sections 7.1.1, 7.1.2 and 7.1.3 respectively.

#### 7.1.1 How accurately do the laboratory results quantify infection?

Accuracy – the closeness of a measured value to the true value – is affected by both the bias and the reliability (or reproducibility) of the measurement technique. Since the quantitative methods described in this thesis are new, and since there is currently no gold standard for quantification of *C. trachomatis* (discussed in more detail in section 7.1.2), any bias inherent in the Q-PCR assay is impossible to assess. Only the reproducibility of the protocol can be formally considered here.

Forty-three people had two conjunctival PCR swabs taken at baseline. Forty-two (98%) of the duplicate swabs returned the same result (by Amplicor) as the primary swab. Four individuals were Amplicor positive for both primary and duplicate swabs; for all four the geometric mean of the numbers of copies of *omp1* estimated on the two aliquots of the duplicate was within one log of the geometric mean of the estimates on the two aliquots of the primary. For eighty-seven of one hundred positive baseline samples, the estimate of the number of copies of *omp1* made for the second aliquot was

within one log of the estimate for the first aliquot. These comparisons suggest a reasonable degree of reproducibility for both the qualitative and quantitative stages of quantification, but do reveal the existence of some variation. Potential sources of this variation, and the measures taken to try and prevent them from affecting the study, are discussed below.

#### **(a) Sample collection**

A detailed protocol for collection of conjunctival swabs was developed and consistently followed for each subject at each time point. Swabs were held and applied in a highly standardised fashion, with the same examiner collecting all swabs taken during the course of the study. Despite this, it is not suggested that an equal number of conjunctival cells or the same volume of tear fluid was collected from each subject, or indeed that it would be possible to achieve perfectly standardised conjunctival sampling using any swabbing technique. Variation between individuals in the amount of tissue collected is unavoidable, because of individual differences in the friability of conjunctivae, and the unpredictable nature of each subject's physical response to swabbing.

To normalise the quantitative results to the volume of tissue sampled, it would be possible to report the load of *C. trachomatis* as the number of copies of *omp1* divided by the number of copies of a single copy human gene present in the same sample. This approach was used by Bièche *et al.* [295], who applied quantitative PCR to the analysis of oncogene amplifications in human breast cancer. Intuitively, however, it seems likely that the ratio of chlamydial to human DNA would be misleading in the present case, since such an index ignores the contribution of both extracellular *C. trachomatis* elementary bodies and accelerated shedding of inflamed tissues [548] to the number of copies of *omp1* present on the swab. Both of these are likely to be important in determining the potential of one eye to become a source of infection for another.

Cross contamination of swabs taken from successive subjects is a potential problem. Much effort was expended in the attempt to prevent it in this study. The balzoi leader who helped steady children for examination and swabbing did not touch subjects' faces. The examiner wore a new pair of latex gloves to examine and swab each subject, and touched neither the outside surface of any pair of gloves nor the glove discard bin, which was opened and closed by a field assistant. Only the examiner touched any part of the swab stick. A second assistant, taking care not to touch the

swab itself, opened the peel-back swab pack to expose the shaft at the end opposite the swab head; the examiner held that end of the swab throughout the procedure. The sterile polypropylene tubes used to transport the swabs were opened by this same assistant, who had been trained not to touch the inside of the tube or cap, and who ensured that her hands remained clean. The swab head and proximal half of the swab shaft was snapped off into the transport tube by bending, rather than by cutting with shears or scissors, the use of which could have provided a route for carry-over contamination. A new cotton bud was used as an aid to lid eversion for each subject. These were handed to the examiner by a third assistant, who did not touch the general waste or glove discard bins, nor handle any material in the post-swabbing sample collection sequence.

#### **(b) Swab storage and transport**

All swabs for PCR were placed on ice in the field, frozen at -20°C within eight hours, and shipped to London on ice or dry ice in the hold of commercial aircraft. At LSHTM, they were stored at -20°C prior to processing. Upon arrival, all swabs inspected had remained frozen during transport. Storage and handling procedures were the same for samples collected at each of the five time points.

#### **(c) Amplicor**

The inclusion of AmpErase in the Amplicor PCR format should eliminate the risk of carry-over amplicon contamination between successive plates. However, cross-contamination of negative samples by positive controls or positive clinical specimens in the same run can be avoided only by good laboratory practice [276]. Standard operating procedures in place in our laboratory (as described in Section 5.7, p.119) were designed with this in mind.

The use of the Amplicor's internal control should have minimised the number of positive samples overlooked because of inhibition of DNA polymerase.

Recently, concern has been expressed over quality control in manufacturing of the NAATs, stemming from occasional imperfect reproducibility noted with Amplicor and other commercial NAAT kits [280]. For example, evaluating cervical specimens by “an antigen detection method”, tissue culture and Amplicor, Peterson *et al.* found that sixteen of 591 patients (2.7%) returned results that were discrepant between those three methods, and that all but one eventual “interpretation change” was due to a change in

Amplior status on repeat testing [277]. Some of these were samples that were Amplior positive on the first test and negative on retesting, while others were initially Amplior negatives that were ultimately shown to be positives: the actual number in each category is difficult to determine from the data given in the paper. What would have been the effect of a similar number of samples being misclassified in the present study? If 2.7% of the Amplior negatives at each time point were actually low copy-number<sup>xxxi</sup> positives (in other words, if the sensitivity of the Amplior kit is 97.3%, rather than the 100% assumed until now in this thesis), twenty-four of 865 (2.7%) baseline negatives in the cohort dataset should have been assigned an *omp1* copy number corresponding to the lowest possible value (4.12) on Q-PCR. This would increase the overall baseline prevalence of ocular CT positivity from 9.5% to 12.0%. The overall COCTL would change from 1.99 (95% CI 1.44 - 2.67) to 2.10 (95% CI 1.54–2.79). Changing a similar proportion of eighteen month negatives (twenty four of 883, 2.7%) to positive would result in the estimated prevalence rising from 0.6% to 3.3%, while the COCTL would increase by a smaller margin – from 0.07 (95% CI 0.01–0.19) to 0.15 (95% CI 0.08–0.27). The magnitude and statistical significance of the fall in the intensity of community infection after antibiotic treatment would be preserved. This scenario probably represents an extreme analysis: the actual proportion of false negatives is likely to have been smaller than 2.7%.

#### (d) Quantitative PCR

Each LightCycler assay used a 4µL aliquot from a total volume of 50µL QIA-amp-eluted DNA. The LightCycler's ability to work with small volumes in glass capillaries is one of its attractions, since this reduces the cost of reagents, speeds thermal cycling, and allows real-time detection of amplified product without tube opening in a true kinetic quantitative PCR format. However, small volumes also increase the effect of sampling variation between aliquots, and – as an extension of that phenomenon in this study – helped increase the chance that samples with small amounts of chlamydial DNA would test positive by Amplior but negative in the LightCycler. Reliable amplification of one copy of *omp1* is suggested by detection of specific product in 65% of seventy-one amplifications of the most dilute standard – an excellent approximation for the

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<sup>xxxi</sup> Schachter and Moncada report that a very small proportion of high-level positive (high signal to cutoff ratio) specimens are not reproducibly positive (by LCx) in their laboratory. However, most of the samples that return variable results in their hands are those with low level positive reactions, which are presumably those with low chlamydial loads [280].



proportion of capillaries prepared with that standard that would be expected to contain at least one copy of the target (63%), based on a Poisson distribution [238, 545]. To compensate for sampling variation between aliquots, two replicate assays were performed in the LightCycler for each Amplicor positive swab, and the geometric mean of the two estimates taken as the swab result. To compensate for the sensitivity advantage of Amplicor over the quantitative assay, the copy number per Amplicor positive, LightCycler negative swab was estimated by maximum likelihood.

#### **(e) Analysis**

In determining the maximum likelihood estimate for the number of copies of *omp1* per Amplicor positive, LightCycler negative swab (Section 5.7.5, p.122), it was assumed that each EB contained four plasmids. This is based on the mean number of plasmids per EB determined by Pickett *et al.* [127] for a laboratory strain of serovar L1 *C. trachomatis*. The actual number of plasmids in any real population of chlamydiae is likely to differ from EB to EB, and to change within an individual organism as it progresses through its life cycle. To date, no formal estimate of the mean number of plasmids per organism has been published for any of the ocular serovars of *C. trachomatis*, or for a wild-type strain of any serovar.

#### **7.1.2 Is quantitative PCR the best way to quantify ocular chlamydial infection?**

This study is the first to use real-time quantitative PCR to explore the epidemiology of ocular chlamydial infections, but not the first to attempt to quantify such infections. The laboratory techniques used in this study build on the work of Bobo [168, 234], Bailey [206], Mahony [225], Huang [238], and their collaborators, amongst many others. Bobo published the first account of the use of PCR to quantify *C. trachomatis*. She coupled thirty cycles of an *omp1*-based PCR to in-solution hybridization with biotin-labelled RNA probes. These were then captured on solid phase and detected by EIA [234]. The test returned more positives than DFA or culture in the evaluation of endocervical specimens [234], and more positives than DFA when testing conjunctival specimens [168]. For the conjunctival swabs, the EIA fluorescent signal strength showed some correlation with the presence or absence of TF and TI [168]. Using the same assay, Muñoz *et al.* found evidence of more intense infection in Tanzanian women with trichiasis than in control women living in the same villages [187]. Unfortunately, the absence of an internal standard (to permit quantification using the competitive route)

and measurement of signal strength only at end point (ruling out kinetic quantification) made this assay only semi-quantitative, as Bobo *et al.* acknowledged [168, 234].

Frost *et al.* [549] developed a semi-quantitative PCR assay for *C. trachomatis* using a competitive format, with primers based on an *omp1* sequence of serovar C. The assay was only semi-quantitative, because quantification of DNA product was achieved by running an aliquot of the amplified product on an agarose gel, staining it with ethidium bromide, and comparing (by inspection) the intensity of the band produced with that generated by PCRs containing known concentrations of a DNA standard. They used this protocol to quantify CT serovars D, E, F, Ia, J and K in twelve cervical and urethral specimens, and compared its sensitivity and reproducibility against that of quantitative culture and direct immunofluorescence. When results were adjusted for the equivalent volume of original sample used in the three techniques, the mean ratio of DNA copies detected to inclusion forming units seen in culture was 595:1, and the mean ratio of DNA copies to immunofluorescent particles visualised at microscopy was 14:1, suggesting that PCR was the most sensitive method. The relationship between Q-PCR and culture was non-linear, though: the number of inclusions seen after three days growth was approximately proportional to the log of the DNA copy number. The reproducibilities of the three methods (as determined by their coefficients of variation<sup>xxxii</sup> on ten replicate determinations on the same sample) were approximately equal.

Eastick *et al.* [550] presented the development of LightCycler-based real-time quantitative PCRs for *C. trachomatis omp1* and plasmid sequences. Testing 501 urine specimens from women attending a genitourinary clinic, they found their Q-PCR to be about as sensitive as the Roche Cobas Amplicor PCR kit. Seven Cobas/LightCycler discrepant specimens were thought to contain chlamydial loads around the detection limit of both assays. In their abstract, the authors mention that the LightCycler assay was able to quantify DNA over a 10,000-fold range, but no data on actual chlamydial loads are included.

Huang *et al.* [238] adapted hot-start PCR to the LightCycler for the detection of chlamydiae. Their SYBR Green assay, upon which the assay used in this thesis was based, was able to demonstrate as few as one copy of the target *omp1* sequence. Using

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<sup>xxxii</sup> The coefficient of variation is the standard deviation of multiple estimates made on aliquots of the same sample, expressed as a percentage of the mean of the estimates. The description given by Frost *et al.* seems to suggest that to determine this coefficient for Q-PCR and the antigen detection method, ten separate aliquots of the same clinical

serial dilutions of *C. psittaci* B577 stock, they were able to demonstrate an approximately linear relationship between the number of copies of chlamydial DNA target and the number of inclusion-forming units in culture.

More recently, other groups have developed quantitative PCR assays and applied them to the study of non-ocular chlamydial infections [551-553] and chlamydial infections in mammals other than man [554, 555]. There appears to be a general consensus that the sensitivity, specificity, dynamic range, rapidity and automation of these tests give them a significant advantage over earlier techniques for quantification of chlamydial loads.

### **7.1.3 What is the clinical and epidemiological significance of high ocular CT loads?**

The fall in the prevalence and intensity of infection in Kahe Mpya following mass treatment with azithromycin was expected. It was not expected that the fall would be sustained for the entire eighteen months of follow-up. One of the consequences of this was to compromise analyses on re-infection. It had been hoped that such analyses could be used to identify load thresholds associated with transmission of infection to family members and other close contacts of infected individuals. In the event, this was not possible, because the prevalence of infection at each post-treatment time point was lower than that at the previous round. The only results from this study, therefore, that contribute to answering the question about the significance of high loads in transmission of *C. trachomatis* are the comparisons of LightCycler and culture data on subjects seen at baseline. For individuals with clinical evidence of active disease, the detection of chlamydial chromosomal DNA in a swab taken from the conjunctiva strongly correlated with the individual being CT culture positive. Culture positivity indicates that viable chlamydiae are being shed, which suggests that the individual is able to infect others. Therefore, if an individual has active disease, and has an ocular infection intense enough for one or more copies of *omp1* to be present per 4µL aliquot after the laboratory extraction protocol described, they should be considered a possible source of infection. Parallel longitudinal studies using Q-PCR conducted at the other sites of the 'Strategies for the control of blinding trachoma' programme may demonstrate higher rates of post-treatment infection and re-infection, and may be able to use quantitative

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sample were tested. For tissue culture, however, on ten separate occasions the number of inclusions seen in one culture were counted by the same observer [549], which is not really an equivalent test of reproducibility.

data to determine which ocular loads of CT DNA are of epidemiological significance. The clinical significance of these measurements should be determined by data showing an association between load over time and progression to conjunctival scarring and trichiasis. Such a study would require long periods of follow-up, possibly without treatment, and might not be ethically acceptable.

## **7.2 RESULTS AGAINST OBJECTIVES**

### **7.2.1 Objective (1): to determine, using quantitative PCR, the ocular load of *C. trachomatis* in different age and gender groups in a community of approximately 1000 individuals before antibiotic treatment**

These data support the hypothesis that children carry the major reservoir of ocular *C. trachomatis*. At baseline, over 90% of the total quantity of ocular chlamydiae detected was found in swabs taken from children less than nine years old. Over 50% of the total burden was in infants under nine months of age. Children are therefore likely to constitute the major source of organism for transmission to others.

The age brackets for fractions-of-total-load at baseline are heavily influenced by two female infants aged eight months and five months, whose swabs together contained nearly 60% of the total community burden of ocular CT. If these two subjects are excluded from the analysis, 50% of the total load is found in residents below four years and two months, and 90% in those aged below nine years and six months; nearly 12% of all ocular *C. trachomatis* is still found in those under one year old. The contribution of under-ones to the ocular CT reservoir is particularly important, as current Tanzanian guidelines exclude children below twelve months of age from receiving the drug as part of mass antibiotic distribution campaigns.

A high prevalence of infection has been reported in infants in other settings. In a trachoma hyperendemic area of Egypt, Schachter and Dawson [330] followed a group of eighty-eight infants from birth to one year of age, taking monthly conjunctival scrapings for evaluation by Giemsa. None of the babies studied had conjunctivitis in the first two weeks of life (suggesting that ocular infection from the mother's genital tract was unlikely), but inclusions were seen from some (number not given) of the one-month old individuals, and forty-eight (55%) of the eighty-eight children had inclusions in one or more of the swabs taken in the first six months of life [330]. Therefore, in the current study, the high prevalence of ocular chlamydial infection seen in infants was not a new finding. The degree of pooling of the ocular load in children under ten years of

age, though, and the finding of the highest-of-all loads in children under one were not expected.

As suggested by Figure 6.1 (p.135), ten years of age was something of a transition point for ocular CT infection, with both the prevalence of infection and the load of *C. trachomatis* being markedly lower in those above than those below that mark. For both males and females, the difference in the COCTL for individuals aged ten years or more versus below ten years was statistically significant (Table 6.13, p.143). In Kahe Mpya in July 2000, if only those aged below ten years (38% of the kitongoji population) had been given antibiotics, more than 97% of the total community ocular CT load would have been covered with either azithromycin or topical tetracycline.

At baseline, there was no consistent trend amongst age groups for males or females to have a higher prevalence or load of infection. In the ten age groups in Table 6.12 (p.142), females had higher average loads than males in four, males had higher average loads than females in five, and in the oldest age group, the average load was essentially equal. There was up to a seven-fold difference in the COCTL between genders within age groups, but these differences were not statistically significant. This reflects the extraordinary range in individual CT loads and the relatively small numbers of individuals in each division, which tends to make the confidence limits for these estimates wide. However, the lack of statistically significant differences in the COCTL between the genders persisted regardless of how age groups were bracketed. Overall, the data do not support the hypothesis that either males or females carry higher infectious loads of ocular chlamydiae in this setting.

The lack of a gender difference in loads is somewhat at odds with expectation. Females typically have a higher burden of clinical signs of disease than males. In central Tanzania, female pre-school children have been noted to have a significantly elevated prevalence of constant, severe, conjunctival inflammation, compared to their male counterparts, and this clinical picture would be expected to correlate with intense infection [357]. Moreover, the difference in the prevalence of clinical signs between the genders generally widens with increasing age. Why did we see no gender difference in quantitative load? Were patterns of clinical disease similar to that seen elsewhere?

The prevalence of active disease (in either or both eyes) in one to nine year-old males in the cohort at baseline was 16/165 (37%) and in one to nine year-old females 79/160 (49%); this difference was statistically significant (z with continuity correction=2.1, two-sided P=0.03). In subjects ten years of age and above, the baseline

prevalences were 13/236 (5%) and 29/360 (8%) for males and females respectively ( $z=1.02$ ,  $P=0.3$ ). At the time of the survey, in Kahe as a whole, twenty-eight of 2434 males examined (1.2%) and forty-eight of 3066 females examined (1.6%) had trichiasis ( $z=1.19$ ,  $P=0.2$ ). These data are in contrast with those obtained in Kongwa, for instance, where women have been noted to have a fourfold increased risk of trichiasis compared to men [331] – a risk ratio that is consistent with that observed in a variety of studies in other endemic areas. It might be speculated that particular social or immunological factors particular to Rombo cause the epidemiology of disease there to differ from the paradigm.

### **7.2.2 Objective (2): to measure, using quantitative methods, the effect of community-wide mass treatment with oral azithromycin 20mg/kg on the ocular load of *C. trachomatis* in different age and gender groups**

The overall community load of ocular *C. trachomatis*, as determined by the COCTL, was 13% of its pre-treatment level two months after mass treatment, 8% at six months, 4% at twelve months, and less than 4% of pre-treatment at eighteen months. The confidence intervals around all four of the follow-up COCTLs suggest that the reduction compared to baseline was statistically significant at each time point, though the decreases seen from two to six months, from six to twelve months, and from twelve to eighteen months were not themselves significant. There were no statistically significant differences in the post-treatment COCTLs between age groups, or between males and females. In other words, decreases in ocular chlamydial load occurred throughout the community. It is possible that the sample size was simply too small to detect a differential effect of treatment by age or gender. However, if there truly is a difference in the efficacy of azithromycin against ocular *C. trachomatis* in different age groups, or in males and females, and a study of nearly 1000 people is not sufficiently large to detect it, it is doubtful that the effect is of sufficient clinical significance to be useful in the planning of trachoma control programmes.

This study was not designed to evaluate the efficacy of azithromycin against clinical trachoma or ocular chlamydial infection, so there was no control group. It is therefore impossible to conclude that the observed reduction in COCTL in Kahe Mpya during the eighteen months after treatment was caused by the intervention alone: a regional secular trend in trachoma [556], enhanced personal hygiene in study participants, or local improvements in environmental factors can not be excluded. No

attempt was made to gather evidence about the first of these potential confounders. If the latter two occurred they were too subtle to have been apparent to the author. One additional contributor to the magnitude of the decrease should be considered: the treatment with topical tetracycline of individuals with active disease at twelve and eighteen months. This will be discussed below, in Section 7.3.4 (p.187).

### **7.2.3 Objective (3): to measure, using quantitative methods, the rate at which infection is re-introduced following community-wide mass treatment with azithromycin**

Infection was not re-introduced to the community for at least eighteen months after mass treatment. Rather, an initial rapid fall in the COCTL after treatment was sustained for at least eighteen months. This sustained reduction in community CT load occurred despite the arrival of 202 new residents. Analysis of the swabs collected from these individuals (taken at the first census review or follow-up point at which they consented to a swab being taken) showed that none of the newborns and only two of the immigrants were Amplicor positive at the time of enrolment. The latter comprised a sixteen year-old girl, who arrived just before the two-month census review and had 15 copies *omp1* in her swab at that time, and a thirteen year-old girl, who arrived just after the five-month census review, and had 19 copies *omp1* in her swab at six months and 162 copies *omp1* in her swab at twelve months. There is no evidence that the contacts of these girls became infected as a result of exposure to them. Ongoing follow-up of the Kahe Mpya cohort, during which ocular CT may return, may provide more data to answer the question of how quickly infection returns to the community.

### **7.2.4 Objective (4): to determine the relative importance of different risk factors in predicting re-emergent infection following community-wide mass treatment with azithromycin**

Because they are based on only a very small number of post-treatment infections, the analyses for risk factors for infection at each post-treatment time point should be interpreted with great caution. Colleagues with more extensive regression modelling experience than the author advise that to avoid statistical instability, at least ten ‘failures’ (in this case, Amplicor positives) are needed for every potential explanatory variable included in the model. This rule of thumb has been ignored here, because adhering to it would have meant examining only age, the exclusion of which – given the

distribution of infection at each time point – would have been difficult to justify, and at most one other variable for two months and six months. Analyses for twelve and eighteen months would have had to have been abandoned, since there were less than ten positives at both of these time points.

Liberal salted with the caveat that it may in fact mislead rather than inform, some cautious commentary on risk factors for post-treatment infection in Kahe Mpya will now be offered. It will be noted that interpretations of the data have been heavily weighted by *a priori* hypotheses about biologically plausible risks.

The association of increasing age with decreasing risk of infection at six months and twelve months is consistent with both the epidemiology of clinical disease and current concepts of the importance of acquired immunity in chlamydial infection [154, 155]. In contrast, at eighteen months, increasing age conferred a very modestly increased risk of being infected. This could be a spurious finding; reflect the beginnings of re-introduction of *C. trachomatis* into the community by two older individuals (who presumably would be more likely than the very young to have had contact with others from outside the village); or (given the low numbers involved) even represent ocular infection with genital strains of *C. trachomatis*. Genotyping of strains collected during the course of this study is underway.

At two months and six months, subjects with visible ocular or nasal discharges were much more likely to be Amplicor positive than those who had no discharge visible on examination. The most probable explanation for these findings is reverse causality – in other words, people who have chlamydial infections of their ocular and nasal epithelia are more likely than uninfected people to produce inflammatory exudates at those sites. However, discharges attract muscid flies, and could provide a means of entry for organism into new hosts: the association may be bi-directional.

In the models generated for two months and eighteen months, access to a latrine was associated with markedly lower risk of infection. At twelve months, paradoxically, all the Amplicor positive individuals had access to a latrine. The interpretation of these data is problematic. Latrine ownership is compulsory in Rombo, and health inspectors impose fines on householders who do not have them, or whose latrines are in a poor state of repair. As a result, many latrines are only for display purposes, while in practice people defaecate in the shamba, ensuring that the pit stays relatively empty and that the enclosure is always clean enough to pass inspection. Because of the low



numbers of positive infections post-baseline, data on variables reflecting whether or not the latrine was actually in use were not analysed.

The output of the twelve month model suggested that people who had received tetracycline at six months were about eleven times as likely to have a CT-positive swab as those who had not. Similarly, at eighteen months, having been treated at twelve months seemed to confer increased risk. These findings probably represent an association between being CT positive at one examination and having had clinically active disease at the previous examination (since it was those with active disease who were given tetracycline), or are simply chance findings. It is possible, but perhaps less likely, that sharing of tubes of tetracycline eye ointment between those to whom it was prescribed and other, infected, symptomatic people, resulted in enhanced transmission of organism within the community.

#### **7.2.5 Objective (5): to trace sources and routes of reinfection with *C. trachomatis* following community-wide mass treatment with azithromycin**

Owing to the small and declining number of positive swabs at each post-treatment time point, tracing sources and routes of 'reinfection' was not possible using data collected in the cohort between baseline and eighteen months after treatment. Ongoing follow-up of this population may provide clues as to the ways in which infection returns to a community after treatment.

#### **7.2.6 Objective (6): to determine the feasibility of using height to guide the dose of azithromycin in a trachoma endemic community in Tanzania**

Using the height-based dosage schedule in Table 6.17 (p.149), and spring scales for individuals assigned by that schedule to '0 tablets', 4379 of 4778 (91.6%) azithromycin-treated people in Kahe would receive either a dose between fifteen and thirty milligrams per kilogram, or the same one gram dose determined by weight (Table 6.19, p.150). Eighteen people would receive a dose in the range 13–15mg/kg, 338 a dose in the range 30–35mg/kg, thirty-eight a dose in the range 35–40mg/kg, and five would receive more than 40mg/kg. The selection of 15–30mg/kg as the safe and effective dose range is arbitrary, and may even be conservative, particularly at the top end. The lower bound, which should be determined by the lowest per-kilogram dose likely to ensure successful treatment whilst avoiding selection of macrolide-resistant pathogens [513], is presently unknown. The upper bound should be calculated to minimise toxic effects. Though

unlicensed for this indication, the recommended dose of azithromycin for typhoid in adults is 500mg daily for seven days. Given azithromycin's prolonged half-life (Section 2.9.3, p.86), it is likely that towards the end of such a course of treatment, the drug is present *in vivo* at concentrations far in excess of that produced by one 20mg/kg dose.

The use of the proposed height schedule (Table 6.17, p.149) for single dose treatment for trachoma would probably be almost as safe and effective as the current weight-based system. In addition, it would lighten the load carried by drug distribution teams, be less prone to equipment malfunction, and be more easily taught to new distributors as trachoma control programmes are progressively expanded to cover more of the total population at risk of trachoma blindness. Height-based treatment should be recommended.

## **7.3 LIMITATIONS OF THIS STUDY**

### **7.3.1 One study site**

This dissertation presents the results of research conducted in a single village in Rombo District, northern Tanzania. Conclusions drawn on the basis of this work should not be assumed to be generally applicable. Unique environmental, social and possibly genetic characteristics of each trachoma endemic area are likely to influence the local distribution of ocular chlamydial infection, effect of mass treatment, and relationship between height and weight in the population. Data presented here, however, have already been compared to data from other trachoma endemic areas in two manuscripts. Both papers suggest that the patterns observed in Rombo may be broadly representative of those that will be found in other communities.

“Strategies for the control of trachoma: an observational study using quantitative PCR” (in press in *The Lancet* [557] at the time of submission of this thesis) examines and compares the pre-treatment distribution of ocular CT load at the three sites of the ‘Strategies for the control of blinding trachoma’ research programme: Kahe Mpya, Maindi sub-village of Kongwa District, central Tanzania, and the Jareng cluster of fourteen villages in the Upper Saloum District, Central River Division, The Gambia. Similar field methods were employed at each site, and laboratory protocols for all samples were as described in this thesis. At all three sites, children had the highest ocular loads of *C. trachomatis*, though the degree of pooling in younger age groups was less marked at the site (Jareng) with the lowest prevalence of active trachoma. There

was no statistically significant difference between the genders in the number of copies of *omp1* found in ocular swabs at any site.

“Antibiotic dosing in trachoma control programs: height as a surrogate for weight in children” was published in the April 2003 issue of *Investigative Ophthalmology and Visual Sciences* [558]. It considers azithromycin dosing by height. Data on height and weight were obtained for a total of 5,558 children aged between six months and fifteen years living in Kahe, Kongwa, Jareng, Malakal (Sudan) and Daboya (Ghana). Using the Kahe dataset as the reference group, this study showed a single height dosing model (more conservative than the one suggested here, but including the use of half tablets) to be adequate for all five populations: more than 97% of children who contributed data to the study would have received between 15mg/kg and 30mg/kg azithromycin using the schedule proposed. Height-based dosing is also supported by a separate study by Basilion *et al.* that used datasets from Vietnam, Tanzania, Ghana and Mali [559].

### **7.3.2 High coverage levels**

In the longitudinal study, 94% of the resident population of Kahe Mpya (the cohort sub-village) received directly-observed azithromycin immediately after the collection of baseline swabs. A further 4% of the population received topical tetracycline for use at home. For a variety of reasons, similarly high coverage levels are not usually achieved in trachoma control programmes. In 2002, for example, antibiotic coverage achieved by the ITI-supported programme in ten treated communities in Rombo ranged between 41% and 59% [560]. Coverage levels reported from other settings are sometimes higher than this (in Ghana: 81% and 85% for 2001 and 2002 respectively [561]), but are generally less than 90%. The impact on the community ocular chlamydial load of achieving only moderate antibiotic uptake can not be estimated purely on the basis of the results from the current study. In fact, there are presently few data that could be used to define a division between coverage levels likely to be effective in reducing the prevalence (or load) of infection, and those likely to be ineffective.

### **7.3.3 Monthly population surveillance**

For logistical reasons, census reviews could not be undertaken more frequently than monthly. As a result, the definition of residency was somewhat exclusive (Section 5.3.2, p.106). Visitors, and even people who considered themselves to be permanent residents but who spent the majority of their time living outside the village were not

included. Many individuals would have spent a few days to weeks in the village during the period of follow-up without being seen by study personnel. A proportion of these people probably had ocular chlamydial infection. Fortunately, the lack of demographic and microbiological information about them can for the most part be overlooked, because imported cases failed to produce an increase in either the prevalence or intensity of infection in the cohort as a whole. In other words, explanations for post-treatment re-infection need not be sought.

#### **7.3.4 Treatment of subjects with clinically active disease at six and twelve months**

Under the terms of the study's ethical approval, it was necessary to provide topical tetracycline to any individual noted to have signs of active disease six or twelve months after mass treatment. At six months, fifty-four people were given tetracycline, including seven of the thirteen (54%) who were subsequently shown to be CT-positive. At twelve months, ninety-four people were given ointment, including five of eight (63%) who were infected. The extent of compliance with the recommended six weeks topical treatment is unknown, but a proportion of the observed longevity of the reduction in COCTL could be ascribed to topical treatment of active cases. An infection-suppressing role for tetracycline is not supported by the increased risk of positivity at twelve and eighteen months in those given ointment at six and twelve months, though doubts about the relevance of this association exist, as has already been discussed. In any event, routine twice-yearly screening of entire communities for active disease as a follow-up to mass treatment is beyond the capacity of most trachoma control programmes, and could not be recommended as an intervention.

### **7.4 IMPLICATIONS FOR TRACHOMA CONTROL PROGRAMMES**

#### **7.4.1 Children are the key demographic group at which to target antibiotics**

Children harbour most of the ocular load of *C. trachomatis* in the community, and should therefore be the principal targets in any population-based antibiotic distribution programme. Because transmission of infection is a dynamic process, and because re-infection after treatment was too infrequent in this study to permit definitive conclusions to be drawn about the risk factors for its occurrence, it is difficult to predict the effect of limiting treatment to children, as compared with the effect of offering treatment to everybody. However, children less than ten years old carried more than 97% of the total ocular CT burden at baseline; if the two infants with the most intense

infections are excluded, children under ten still contribute nearly 93% of the remaining community load. At two, six, twelve and eighteen months after mass treatment, children below ten had 94%, 99%, 66%, and nearly 100% respectively of the total burden of ocular chlamydiae in Kahe Mpya, while making up only 38% or 39% of the total population of the sub-village at any time point. Studies that will empirically determine the differential effect between mass treatment and treatment only of children may be worth conducting, though implementation of an under-tens-only policy might well be unpopular in treated communities. Adults who have previously taken azithromycin are generally enthusiastic about the prospect of receiving it again. For communities that have no prior experience with the drug, without concerted efforts to involve opinion leaders in decision making, treatment only of children might be mistrusted.

The two highest individual CT loads were found in girls aged eight months and five months respectively. There is no reason to suspect that these two were ‘outliers’, in the sense of their swab results being erroneous. Current guidelines that bar distribution of azithromycin to infants should be re-evaluated in the light of these findings, particularly as there are currently no data suggesting that the drug is unsafe in this age group, and good data to suggest that it is safe in pregnancy (Section 2.9.9(a), p.91).

There were no significant differences in the prevalence or load of infection between the genders. On the basis of the data presented here, there is no rational basis for restricting treatment to either males or females in any age category. In particular, the Tanzanian recommendation that, in villages in which the prevalence of active disease is 10-20%, all children and adult women, but not adult men, be treated receives no support.

#### **7.4.2 Active disease predicts CT load, but not so well as to justify examination of whole endemic communities**

Particularly as the prevalence of trachoma declines, some control programmes begin to favour targeted treatment of individuals with active disease over mass treatment of all members of affected communities. At baseline, use of this approach would have resulted in treatment of 98% of the total community load of *C. trachomatis*. Why, then, has the primary focus in this dissertation been on loads in demographic, rather than clinical groups? There are three reasons. First, examination usually requires skilled ophthalmic personnel. These people are in short supply in most trachoma

endemic areas, and the demand for their services is high. If less highly trained graders are used, poor diagnostic accuracy and reliability could mismatch treatment with need, destroying any potential advantage of the targeted strategy. Selecting (say) all children under ten as the treatment target group is easier than selecting all people with TF and/or TI according to the strict WHO-defined criteria. Second, screening whole communities is very time consuming. Treating all children under ten in the cohort would have required zero examinations and the dispensing of 360 doses of azithromycin. Treating all those with TF and/or TI would have required 956 examinations and 174 doses of azithromycin, with (presumably) an equivalent impact on community levels of infection, given the relative proportion of the total community CT load found in each of these population subsets. Third, after a single round of treatment, children under ten years of age had a higher fraction of the total community ocular CT load at each time point than did the group of individuals with signs of active disease (compare Section 7.4.1, p.187, with Table 6.32, p.171). In this setting, therefore, directing treatment at children rather than people with active disease would have been more time-efficient at baseline, and (had re-treatment of the entire community been scheduled at any post-baseline time), it would still have been more efficient to use an age-directed than disease-directed strategy.

### **7.4.3 High coverage may produce a sustained impact on community load**

In this study, high coverage mass azithromycin treatment of a trachoma endemic community was associated with a substantial decrease in both the prevalence and intensity of ocular *C. trachomatis* infection. The decrease was sustained for at least eighteen months after treatment, perhaps assisted by distribution of tetracycline eye ointment to individuals with clinically active trachoma at six and twelve months. Because of the lack of a control group, the evidence is only circumstantial, but nevertheless suggests that the potential efficacy of mass azithromycin treatment may be far more dramatic than had previously been supposed. As already indicated, similarly high coverage levels and six-monthly individual re-examinations are unlikely outside the research setting. Further investigations of the effect of mass treatment on infection in other communities where uptake of antibiotics is lower would help to determine necessary coverage levels for best impact, and whether in fact there is a threshold coverage that will lead, as may have occurred in Kahe Mpya, to a permanent suppression of ocular chlamydial infection in the population. In the absence of real data

comparing coverage with effect, it is probably not drawing too long a bow to suggest that the higher the coverage, the greater the reduction in CT load.

#### **7.4.4 Height-based treatment is likely to be safe and effective**

Azithromycin is virtually the ideal antibiotic for the treatment of trachoma. It is effective in single dose, extremely well tolerated, generally popular amongst recipients, and – thanks to the generosity of its manufacturer – is provided free to a number of control programmes. The drug works and is available, but in the field, some method for determining how much of the drug is to be given to each individual lining up to receive it is required. Standard weighing scales become unreliable when transported over rough roads and used outside in dusty or wet conditions. The relatively robust electronic scales used for this research project were expensive, and hundreds would be needed to outfit a national azithromycin distribution programme. A better option would be to use height sticks or tapes to determine the number of tablets required, and spring scales (which are durable, portable, and available for approximately £15 each from TALC) to weigh children who need azithromycin suspension, provided of course that height-based determination of tablet dose is safe and effective. It is, according to the data generated here. The argument behind this assertion was presented in Section 7.2.6 (p.184).

### **7.5 SUGGESTIONS FOR FURTHER RESEARCH**

(1) Members of the cohort enrolled in the longitudinal study in Kahe Mpya have been enthusiastic partners in this research, and represent a willing, useful resource for ongoing studies of the epidemiology of ocular chlamydial infection. Under the terms of the ethical approval obtained for the project, re-treatment of Kahe village with azithromycin was required at two years. However, all consenting residents were examined and swabbed prior to that treatment round, and follow-up will continue at six-monthly intervals for at least another eighteen months.

(2) Given the high loads noted in infants in this project, studies to establish the safety of azithromycin in children below the age of six months are required. There are currently no data that suggest that the use of the drug in this age group should continue to be contraindicated.

(3) Mass treatment with azithromycin has been associated with increased genotypic variation of ocular *C. trachomatis* isolates. In the Tanzanian arm of the ACT trial, one year after mass treatment, the proportion of infected individuals whose CT isolates had amino-acid-sequence-altering nucleotide substitutions in *omp1* (compared to baseline genotypes) was higher in azithromycin than tetracycline treated subjects [562]. Whether this reflects the introduction of new isolates from untreated immigrants or *de novo* selection of mutants is not known. Alteration in the primary structure of MOMP could potentially lead to immunoevasion. Further studies are warranted. We are currently undertaking full length sequencing of *omp1* from all positive samples collected during the course of the longitudinal study.

(4) Similarly, research into the potential for selection of macrolide resistance in *C. trachomatis*, *S. pneumoniae*, and other human pathogens by the use of azithromycin for trachoma control should be a priority. The establishment of regional sentinel sites with access to -70°C freezers or liquid nitrogen facilities (to preserve samples for chlamydial culture and sensitivity determination) may be the best way to undertake this kind of monitoring.

(5) Ongoing work at the other two sites of the ‘Strategies for the control of blinding trachoma’ programme may help to discover contact chlamydial loads and kinds of exposure that constitute a risk for transmission of infection.

(6) Inexpensive, locally acceptable, sustainable strategies for achieving high coverage in azithromycin distribution campaigns should be sought. Community directed distribution, as deployed in the African Programme for Onchocerciasis Control, may be worth evaluating.



## APPENDIX 1: CORRELATION OF LABORATORY TESTS FOR *C. TRACHOMATIS* INFECTION AND CLINICAL SIGNS OF ACTIVE TRACHOMA

Table A1.1: Published data on the correlation of laboratory tests for ocular *C. trachomatis* infection with clinical signs of active trachoma

Country	Selection of subjects	Background prevalence of trachoma	Test	Prevalence of positive tests	Ref.
Egypt	Children 1–10 years of age with at least moderately severe active trachoma, screened at a medical clinic	“Holoendemic”	Giemsa	14% (13/91)	[217]
Nepal	All children aged 1–10 years from 6 villages examined; Giemsa staining performed on 15 children arbitrarily selected from those with active disease	46/726 (6%) had active disease	Giemsa	0% (0/15)	[311]
Kenya	Children with abnormal ocular discharge visiting a health centre after an invitation was issued for families with eye problems to attend	90% of persons <i>in the district</i> had “past or current findings suggestive of trachoma” in a survey performed 7–9 years before the study	Culture	34% (31/91) of those with 3 or more follicles 13% (17/130) of those with papillary hypertrophy sufficient to obscure underlying blood vessels, but 2 or fewer follicles	[103]
Egypt	Children 1–10 years of age with at least moderately severe active trachoma, screened at a medical clinic	“Holoendemic”	Culture	37% (34/91)	[217]
The Gambia	All residents of one village (n~900) examined in 1984; swabs taken from everyone with active disease and a random sample of those without active disease	Same population as in Mabey <i>et al.</i> (1987) [253]; prevalence data provided in this paper only as a graph of prevalence against age for those 0–18 years old in 1984	Culture	1% (1/97) of those without active disease 43% (31/72) of those with active disease	[171]

The Gambia	All residents of one village (n~900) examined twice: once in 1985, once in 1986. In 1985 swabs collected from a randomised, age-stratified sample of the population; in 1986 only from clinically active cases	22% of those aged ≥15 had active disease.	Culture	3% (3/90) of those with insignificant or no disease 18% (30/169) of those with active disease	[253]
Nepal	All children aged ≤5 years in three villages of Lumbini Zone, Western Nepal (n=430); specimens for 400 subjects reported	Moderate to severe intensity of inflammation in 85/430 (21%) examined	Culture (McCoy cells) Serial passage after 96 hours on 2 duplicate plates if negative on initial inoculation	44/400 (11%) ~5% of 267 with no active disease ~13% of 54 with mild active disease ~11% of 18 with moderate active disease ~31% of 61 with severe active disease (read from graph)	[222]
Tanzania	Stratified random sample of 20 villages drawn. Within each village, a cluster sample of children aged 1–7 and their mothers or female caretakers examined; swabs taken from all those examined in the first 9 villages (n=1671)	589/1090 (54%) of children aged 1–7 had active trachoma 52/581 (9%) of women had active trachoma	Culture Considered inadequate if cell monolayer completely destroyed in both 1 <sup>st</sup> and 2 <sup>nd</sup> passage (n=23) or if it had been completely destroyed in one and partially destroyed in the other (n=12)	4% (34/927) of those with no sign of trachoma 31% (150/481) of those with TF only 51% (73/142) of those with TI 9% (7/77) of those with TS only 11% (1/9) of those with TT without TF or TI	[548]
USA	Students aged 12 to 21 attending the Stewart Indian School near Carson City, Nevada. All were American Indians from reservations in the southwestern USA	“Highly endemic”	In house IF of conjunctival scrapings using polyclonal fluorescein-labelled serum from a rabbit immunised with a yolk sac-grown strain of TRIC agent	9/17 (53%) with clinical signs of active disease (based on severity of purulent discharge, follicular or papillary hypertrophy, pannus, and conjunctival and corneal scarring) in 1965 8/15 (53%) with clinical signs of active disease in 1966 3/5 (60%) with clinical signs of active disease in 1967 5/29 (17%) without clinical signs of active disease in 1965 11/31 (35%) without clinical signs of active disease in 1966 15/41 (37%) without clinical signs of active disease in 1967	[180, 243]

Kenya	Children with abnormal ocular discharge visiting a health centre after an invitation was issued for families with eye problems to attend	90% of persons <i>in the district</i> had past or current findings suggestive of trachoma	DFA (MicroTrak) Slides considered (+) if =10 EBs seen	17/60 (34%) with 3 or more follicles 13/95 (14%) with papillary hypertrophy sufficient to obscure underlying blood vessels, but 2 or fewer follicles	[103]
Egypt	Children 1–10 years of age with at least moderately severe active trachoma, screened at a medical clinic	“Holoendemic”	DFA (MicroTrak) Slides considered (+) if =10 EBs seen	38% (35/91)	[217]
Nepal	All children aged =5 years in three villages of Lumbini Zone, Western Nepal (n=430); specimens for 400 subjects reported	Moderate to severe intensity of inflammation in 85/430 (21%) examined	DFA (MicroTrak) Slides considered (+) if =10 EBs seen	33/400 (8%) ~15% of 267 with no active disease ~28% of 54 with mild active disease ~22% of 18 with moderate active disease ~34% of 61 with severe active disease (read from graph)	[222]
Tanzania	One index child from each of 234 households with children aged 1–7, randomly selected from a village with no previous trachoma control programme	137/234 (58.5%) active disease in those examined	DFA (MicroTrak) Slides considered (+) if =5 EBs seen.	1/97 (1%) without TF or TI 28/100 (28%) with TF but not TI 22/37 (60%) with TI ± TF	[168]
Tanzania	Stratified random sample of 20 villages drawn. Within each village, a cluster sample of children aged 1–7 and their mothers or female caretakers examined; swabs taken from all those examined in the first 9 villages (n=1671)	589/1090 (54%) of children aged 1–7 had active trachoma 52/581 (9%) of women had active trachoma	DFA (MicroTrak) Slides considered (+) if =5 EBs seen. Slides considered inadequate if <200 epithelial cells seen: 188 slides were inadequate, but 2 of these had >10 EBs and were included.	6% (48/813) of those with no sign of trachoma 49% (221/447) of those with TF only 71% (100/140) of those with TI 21% (16/76) of those with TS only 11% (1/9) of those with TT without TF or TI	[548]
The Gambia	All residents of two trachoma endemic villages (n=1363) examined; swabs taken from 1348 subjects for EIA	201/1348 (15%) in those swabbed for EIA	EIA (Novo Nordisk)	25% (66/126) of those with mild active disease 37/47 (79%) with moderate active disease 22/28 (79%) with severe active disease 75/1147 (6.5%) without active disease	[206]

The Gambia	All residents of one village (n~900) examined twice: once in 1985, once in 1986. In 1985 swabs collected from a randomised, age-stratified sample of the population; in 1986 from the whole population	22% of those aged $\geq 15$ had active disease.	EIA-PCE (Boots Celltech)	56/228 (25%) with active disease 49/997 (4.9%) with insignificant or no disease 10/49 (20%) with severe scarring (C3)	[253]
Egypt	Children 1–10 years of age with at least moderately severe active trachoma	“Holoendemic”	EIA (Chlamydiazyme)	36/91 (40%)	[217]
The Gambia	All inhabitants of one village (n~950) examined in 1990; swabs taken from all those with active disease and from all members of two households, one containing substantial numbers of trachoma cases, the other free of clinical trachoma	96/844 inhabitants of the village had clinically active trachoma	EIA (Novobiolabs)	0/37 without active disease 10/12 (83%) with severe active disease 8/28 (29%) with moderate disease 6/56 (11%) of those with mild disease	[240, 272]
The Gambia	All residents of one village (n~900) examined in 1984; swabs taken from everyone with active disease and a random sample of those without active disease	Same population as in [253]; prevalence data provided in this paper only as a graph of prevalence against age for those 0–18 years old	Tear MIF – ELISA for sIgA	38/68 (56%) of those with active disease 10/23 (43%) of those without active disease but with scarring	[171]
Kenya	Children with abnormal ocular discharge visiting a health centre after an invitation issued for families with eye problems to attend	90% of persons <i>in the district</i> had “past or current findings suggestive of trachoma” in a survey performed 7–9 years before the study	Serology (serum MIF for IgM and IgG) Positive result defined as an antibody titre of $\geq 1:8$ .	32/39 (82%) with 3 or more follicles 18/47 (38%) with papillary hypertrophy sufficient to obscure underlying blood vessels, but 2 or fewer follicles	[103]
Nepal	All children aged $\geq 5$ years in three villages of Lumbini Zone, Western Nepal (n=430); specimens for 400 subjects reported	Moderate to severe intensity of inflammation in 85/430 (21%) examined	In house DNA probe using radiolabelled cloned plasmid DNA from serovar C strain TW3	70/400 (18%) ~10% of 267 with no active disease ~22% of 54 with mild active disease ~22% of 18 with moderate active disease ~46% of 61 with severe active disease (read from graph)	[222]

The Gambia	All residents of two trachoma endemic villages (n=1363) examined; swabs taken from 1332 subjects for PCR	200/1332 (15%) active disease in those swabbed for PCR	In house PCR against pCT	83/128 (65%) with mild active disease 38/44 (86%) with moderate active disease 23/28 (82%) with severe active disease 85/1132 (7.5%) without active disease	[206]
The Gambia	All inhabitants of one village (n=950) examined in 1990; swabs taken from all those with active disease and from all members of two households, one containing substantial numbers of trachoma cases, the other free of clinical trachoma	96/844 inhabitants of the village had clinically active trachoma	In house PCR against MOMP	2/37 (5%) without active disease 8/12 (67%) with severe active disease 16/28 (57%) with moderate disease 25/56 (45%) with mild disease	[240, 272]
Tanzania	One index child from each of 234 households with children aged 1–7, randomly selected from a village with no previous trachoma control programme	137/234 (58.5%) active disease in those examined	In house PCR-EIA against MOMP	23/97 (24%) without TF or TI 54/100 (54%) with TF but not TI 35/37 (95%) with TI $\pm$ TF	[168]
Tanzania	4932 women aged 18 years or older from 11 villages examined in 1989; follow-up examination planned for all women with scars living in 6/11 villages and a random sample of women without scars from the same villages. 523/745 with scars examined; 503/749 without scars examined.	147/1014 active disease in those examined	In house PCR-EIA against MOMP	17/453 (3.8%) without scars at baseline or active disease at F/U 5/16 (31.3%) without scars at baseline, with TF (but not TI?) at F/U 14/32 (43.8%) without scars at baseline, with TI at F/U 24/414 (5.8%) with scars at baseline, without active disease at F/U 2/6 (33.3%) with scars at baseline, with TF (but not TI?) at F/U 34/93 (36.6%) with scars at baseline, with TI at F/U 36/499 (7.2%) without scars at baseline, without TT at F/U 0/2 (0%) without scars at baseline, with TT at F/U 51/466 (10.9%) with scars at baseline, without TT at F/U 9/47 (19.2%) with scars at baseline, with TT at F/U	[187]

Tanzania	All residents of one subvillage invited (956 of 978 residents examined)	174/956 (18%) active disease	PCR (Amplicor)	17/63 (27%) with TF without TI 10/56 (18%) with TI without TF 30/55 (55%) with TI and TF 2/86 (2%) with TS but neither TF nor TI 31/696 (4%) with no sign of trachoma	[563]
Egypt	All inhabitants of two trachoma endemic villages	46.3% active disease in 0–10 years old 3.6% active disease in >10s	LCR (LCx)	382/1330 (28.7%) without active trachoma 84/331 (25.4%) with mild follicular trachoma (F1, P1, P2) 176/288 (61.1%) with follicular trachoma only (F2, F3) 97/120 (80.8%) with severe inflammatory trachoma (P3)	[169]
The Gambia	All inhabitants of several trachoma endemic villages	34% active disease in 0–10 years old 4.1% active disease in >10s	LCR (LCx)	241/990 (24.3%) without active trachoma 221/480 (46.0%) with mild follicular trachoma (F1, P1, P2) 81/155 (52.3%) with follicular trachoma only (F2, F3) 85/122 (69.7%) with severe inflammatory trachoma (P3)	[169]
Tanzania	All inhabitants of two trachoma endemic villages	60.1% active disease in 0–10 years old 17.2% active disease in >10s	LCR (LCx)	22/1235 (1.8%) without active trachoma 61/574 (10.6%) with mild follicular trachoma (F1, P1, P2) 169/408 (41.4%) with follicular trachoma only (F2, F3) 239/436 (54.8%) with severe inflammatory trachoma (P3)	[169]
Nepal	17 of 18 wards in two village development committees were separated into randomisation units of 1, 2 or 3 wards. 50 randomly chosen children aged 1–7 were chosen from each unit; LCR performed on all the sampled clinically active cases (n=117) and on a randomly chosen 118 normals	A pre-treatment survey of 1597 children aged 1–10 in 5 arbitrarily chosen wards showed a prevalence of active disease of 28.5%.	LCR (LCx)	29/117 (25%) of clinically active cases 5/118 (4%) of clinically normal cases	[495]
Nepal	All children aged 1–10 years from six villages examined; swabs taken from all those with active disease (TF or TI) and one eighth of those without active disease (selected at random)	46/726 (6%) active disease	LCR (LCx)	0/46 (0%) with active disease (TF and/or TI in the swabbed eye) 0/44 (0%) without active disease	[311]

Egypt	All inhabitants of two villages (n=2067)	408/2067 (19.7%) active disease	LCR (LCx)	465/1659 (28%) without active disease 176/288 (61%) with TF but not TI 97/120 (81%) with TI	[207]
Australia	Children aged <15 from one Aboriginal community (n=221) screened for trachoma. Of those with follicular trachoma (n=?), 48 had conjunctival swabs taken	Not specified. In “many Aboriginal communities in central Australia ... 20% of children (have) characteristic eyelid inflammation”	LCR (LCx)	8/48 (17%) with TF	[309, 497]
China	All individuals (any age) with clinically active trachoma (TF or TI) in seven villages randomly selected from Dongfang District, Hainan Province. No two cases were from the same household.	Prevalence in 1–7 year old children 2%. Sample age range 2–75 years; though 21/25 swabs were from children <10	LCR (LCx)	2/25 (8%)	[326]
Nepal	55 children aged 1–10 were randomly selected from each of 4 villages in which the prevalence of active trachoma was found to be <10%. Swabs taken from selected children who were clinically active	7% in 1–10 year-old children	LCR (LCx)	2/24 (8%)	[326]
Nepal	55 children aged 1–10 were randomly selected from a village in which the prevalence of active trachoma was found to be >30%. Swabs taken from selected children who were clinically active	39% in 1–10 year-old children	LCR (LCx)	15/24 (63%)	[326]
Australia	Children aged <15 from one Aboriginal community (n=221) screened for trachoma. Of those with follicular trachoma (n=?), 48 had conjunctival swabs taken	Not specified. In “many Aboriginal communities in central Australia ... 20% of children (have) characteristic eyelid inflammation”	PCR (Cobas Amplicor)	3/48 (6%) with TF	[309, 497]

Australia	Children aged <15 from one Aboriginal community (n=221) screened for trachoma. Of those with follicular trachoma (n=?), 48 had conjunctival swabs taken	Not specified. In “many Aboriginal communities in central Australia ... 20% of children (have) characteristic eyelid inflammation”	PCR (In house; target gene not specified)	7/48 (15%) with TF	[309, 497]
Australia	Children aged <15 from one Aboriginal community (n=221) screened for trachoma. Of those with follicular trachoma (n=?), 48 had conjunctival swabs taken	Not specified. In “many Aboriginal communities in central Australia ... 20% of children (have) characteristic eyelid inflammation”	Hybridization probe (Gen-Probe Pace 2)	0/48 (0%) with TF	[309, 497]



## **APPENDIX 2: FORMS**

---

**Survey form (a)**

Date: \_\_\_\_ / \_\_\_\_ / \_\_\_\_

FA: \_\_\_\_

Name of head of balozi: \_\_\_\_\_

Name of head of kaya: \_\_\_\_\_

Balozi / kaya:

☐ ☐ ☐ ☐

B/K number painted? (Y/N):

☐

Draw a map of the kaya here.

Room number	Roof type (T=tin, S=straw, N=none)

Structure	N	E	# sats	Time

Survey form (b) - consent (English)

Balozi / kaya:

Page  of

The information sheet concerning this study has been read to me, and I understand what will be expected of me if I take part in this study.

My questions concerning this study have been answered by \_\_\_\_\_. I understand that participation in this study is voluntary. I also understand that I may withdraw from this study at any time without giving a reason and that this will not affect my normal care.

I agree to take part in this study.

#	Name of person for whom consent given	Name of person giving consent	Relationship	Signature/thumbprint	Date	Witness*	Date

\* Witness: By signing in this column I warrant that I have read this form and the information form to the persons against whose names my signature appears. I am sure that each of these persons has understood what is required of him/her and has agreed to take part in the study.

## Survey form (b) - ridhaa (Kiswahili)

Balozi / kaya:

Ukurasa  wa

Karatasi ya maelezo kuhusiana na utafiti huu nimesomewa, na ninafahamu ninategemewa kufanya nini ikiwa nitashiriki kwenye utafiti huu.

Maswali yangu kuhusiana na utafiti huu ya mejibiwa na \_\_\_\_\_. Ninafahamu kwamba kushiriki katika utafiti huu ni hiari yangu. Pia ninafahamu kwamba ninaweza kujiondoa kwenye utafiti wakati wa wote na kwamba kufanya hivyo hakutaathiri matibabu yangu ya kawaida.

Nakubali kushiriki kwenye utafiti huu.

#	Jina la mtu aliyetolewa ridhaa	Jina la mtu anayetoa ridhaa	Uhusiano	Sahihi / alama ya dole	Tarehe	Shahidi*	Tarehe

\* Shahidi: Kwa kuweka sahihi kwenye safu hii ninathibitisha kwamba nimesoma hii fomu na fomu ya maelezo kwa hao watu ambao majina yao yana sahihi yangu. Nina hakika kwamba kila mmoja wa watu hawa ameelewa anatakiwa kufanya nini na amekubali kushiriki katika huu utafiti.

Survey form (c) (Front)

Balozi / kaya:

Page  of  FA: \_\_\_\_\_

Date of 1<sup>st</sup> survey visit: \_\_\_\_ / \_\_\_\_ / \_\_\_\_

Name of head of kaya: \_\_\_\_\_

Examiner: \_\_\_\_\_

#		Name	C	Alias	Date of birth	Sx	Eth		Edu	Sh now		E d/c	N d/c	[right eye]					[left eye]					Fly eye
														T T	C O	T F	T I	T S	T T	C O	T F	T I	T S	

Comments: \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Survey form (c) (Back: treatment form for non-cohort mabalozi)

#		Tarehe	??		Uzito			Tab	mL	Urefu			Comments
Totals this page													

Comments: \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Data recorder: \_\_\_\_\_ Weight and height: \_\_\_\_\_ Azithromycin: \_\_\_\_\_

# Survey form (d) (English)

Date: \_\_\_\_ / \_\_\_\_ / \_\_\_\_ FA: \_\_\_\_\_

Name of balozi leader: \_\_\_\_\_

Name of head of household: \_\_\_\_\_

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
--------------------------	--------------------------	--------------------------	--------------------------

How many cattle stay in the shamba around this house overnight?				(1)
How many cattle does the family own in total (both here and elsewhere)?				(2)
How many sheep do the family own in total?				(3)
How many goats do the family own in total?				(4)
How many ducks do the family own in total?				(5)
How many chickens do the family own in total?				(6)
How many dogs live with this family?				(7)
How many pigs do the family own in total?				(8)
How many rabbits do the family own in total?				(9)
How many pigeons do the family own in total?				(10)
How many minutes does it take to walk from the house to the place where water is collected in the wet season?				(11)
How many times a week are you able to collect water in the wet season?				(12)
How many minutes does it take to walk from the house to the place where water is collected in the dry season?				(13)
How many times a week are you able to collect water in the dry season?				(14)
Do the family have access to a latrine? (No=0, Family use only=1, Shared=2, Refused to answer=5)				(15)
If the family has access to a latrine, ask if you can go and see it. Is there a beaten path to the door? (No=0, Yes=1, Not applicable [no latrine]=3, Refused to allow inspection=5)				(16)
Is there adequate screening of the latrine for privacy? (No=0, Yes=1, Not applicable [no latrine]=3, Refused to allow inspection=5)				(17)
Are there flies around the latrine? (No=0, Yes=1, Not applicable [no latrine]=3, Refused to allow inspection=5)				(18)
What kind of floor does the latrine have? (None=0, Sticks=S, Wood=W, Cement=C, Another type=A, Not applicable [no latrine]=3, Refused to allow inspection=5)				(19)
When you look into the latrine with a torch, can you see faeces? (No=0, Yes [top more than 60cm below ground level]=1, Yes [top less than 60cm below ground level]=2, Not applicable [no latrine]=3, Refused to allow inspection=5)				(20)

# Survey form (d) (Kiswahili)

Tarehe: \_\_\_\_ / \_\_\_\_ / \_\_\_\_ FA: \_\_\_\_

Jina la balozi: \_\_\_\_\_

Jina la mkuu wa kaya: \_\_\_\_\_

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
--------------------------	--------------------------	--------------------------	--------------------------

Ng'ombe wangapi wanaishi hapa?				(1)
Ng'ombe wenu jumla ni wangapi pamoja na walio kwa watu?				(2)
Kondoo wenu jumla ni wanngapi?				(3)
Mbuzi wenu jumla ni wangapi?				(4)
Bata wenu jumla ni wangapi?				(5)
Kuku wenu jumla ni wangapi?				(6)
Mbwa wangapi wanaishi pamoja na nyinyi?				(7)
Nguruwe wenu jumla ni wangapi?				(8)
Sungura wenu jumla ni wangapi?				(9)
Njiwe wenu jumla ni wangapi?				(10)
Mnachukua dakika ngapi toka hapa mpaka mahali pakuteka maji wakati wa masika?				(11)
Mnapata maji kwenye bomba mara ngapi kwa wiki wakati wa masika?				(12)
Mnachukua dakika ngapi toka hapa mpaka mahali pakuteka maji wakati wa kiangazi?				(13)
Mnapata maji kwenye bomba mara ngapi kwa wiki wakati wa kiangazi?				(14)
Je, mna choo? (Hamna=0, Kwa matumizia nyumba hii tu=1, Tunachangia=2, Wamekataa kujibu=5)				(15)
Kama kuna choo, waombe kama unaweza kukiangalia. Je, kuna kichochoro cha njia kwenda chooni? (Hamna=0, Ndiyo=1, Haipo [hakuna choo]=3, Wamekataa kukuruhusu kukagua=5)				(16)
Ina uzuio wa kutosha mtu asionekane? (Hamna=0, Ndiyo=1, Haipo [hakuna choo]=3, Wamekataa kukuruhusu kukagua=5)				(17)
Kuna inzi karibu na choo? (Hamna=0, Ndiyo=1, Haipo [hakuna choo]=3, Wamekataa kukuruhusu kukagua=5)				(18)
Choo kina sakafu ya aina gani (Hamna=0, Miti [Sticks]=S, Mbao [Wood]=W, Sementi [Cement]=C, Nyingine [Another]=A [tafadhali taja sawasawa], Haipo [hakuna choo]=3, Wamekataa kukuruhusu kukagua=5)				(19)
Ukimwulika kwa torchi ndani ya choo, unaweza ukayaona mavi? (Hapana=0, Ndiyo [kama kimejaa na kubakiza zaidi ya sentimeta sitini toka usawa wa arthi]=1, Ndiyo[kama kimejaa na kubakiza chini ya sentimeta sitini toka usawa wa arthi]=2, Haipo [hakuna choo]=3, Wamekataa kukuruhusu kukagua=5)				(20)





## APPENDIX 3: CODES

Table A3.1: Codes for recording demographic details

Field	Abbreviation on form	How recorded on the form
Mwenyeji number	#	<p>There are two boxes on the form on the form for each mwenyeji. On the first survey form (c) for any household, fill in these boxes from the top of the form to the bottom with the numbers 1 to 10, like this:</p> <div style="display: flex; align-items: center; justify-content: center;"> <div style="border: 1px solid black; padding: 2px 5px; margin: 0 5px;">0</div> <div style="border: 1px solid black; padding: 2px 5px; margin: 0 5px;">1</div> <span style="margin: 0 10px;">to</span> <div style="border: 1px solid black; padding: 2px 5px; margin: 0 5px;">1</div> <div style="border: 1px solid black; padding: 2px 5px; margin: 0 5px;">0</div> </div> <p>If there is more than one page, the first mwenyeji on the second page should be given the number 11, the next one 12, and so on.</p>
First and last name	Name	Written in capital letters using standard alphabet
Room (chumba) number	C	The room number (as shown on the map of the compound) in which that person sleeps at night.
Alias (nicknames)	Alias	Written in capital letters using standard alphabet
Date of birth (DOB)	Date of birth	<p>The person's date of birth written in the format</p> <div style="border: 1px solid black; padding: 5px; display: inline-block; margin-bottom: 10px;">             day / month / year           </div> <ul style="list-style-type: none"> <li>• Write the year in full (for 6<sup>th</sup> February 1975, write 06/02/1975, rather than 06/02/75)</li> <li>• The best estimate of the individual or their family will be acceptable, though if some kind of official record (eg vaccination card, birth certificate) is available, it should be used.</li> <li>• If the person gives you their age rather than their date of birth, look at the ages to dates of birth conversion table to determine the year of birth.</li> <li>• If an adult is unsure of their age and date of birth, use the local events calendar to determine the approximate year of birth.</li> <li>• If you can only determine the year of birth but not the month or day, record DOB as the first of January in that year (01/01/yyyy). If you can determine the year and month but not the day, record DOB as the first of that month (01/mm/yyyy).</li> </ul>
Sex	Sex	1 = Male/mwanaume 2 = Female/mwanamke 8 = Not known
Ethnic group	Eth	61 = Chagga 62 = Kamba 63 = Masai 64 = Kikuyu 65 = Mpare 66 = Msambaa

		67 = Msukuma 68 = Wameru 69 = Wairak 70 = Mang'ati 87 = Other (tafadhali taja sawasawa - andika 'comments') 88 = Not known
Highest level of formal education <i>completed</i>	Edu	00 = no formal education/did not finish standard 1 01 = standard 1 02 = standard 2 03 = standard 3 04 = standard 4 05 = standard 5 06 = standard 6 07 = standard 7 08 = standard 8 (Kenya only) 09 = standard 9 10 = standard 10 11 = standard 11 12 = standard 12/form four 13 = standard 13/form five 14 = form six 15 = college or university 88 = not known
School <i>currently</i> attended	Sh now	00 = not at school 31 = Kiura Primary 32 = Kahe Primary 33 = Mtikome Primary 34 = Mamonjo Primary 35 = Kwalamahondo Primary 36 = Usseri Primary 37 = Mreyai Primary 38 = Reha Primary 39 = Kingachi Primary 40 = Nesae Primary 41 = Matolo Secondary 42 = Kirongo Secondary 43 = Tarakea Secondary 44 = Mamtukuna Folk Development College 88 = not known

Table A3.2: Codes for facial cleanliness variables

Variable	Abbreviation	Codes for recording / data entry
Pus or dried secretions around one or both eyes	E d/c	No=0 Yes=1 Refused to allow inspection=5 Already examined elsewhere=7
Visible discharge from the nose	N d/c	No=0 Yes=1 Refused to allow inspection=5 Already examined elsewhere=7
Flies seen in contact with the eye	Fly Eye	No=0 Yes=1 Refused to allow inspection=5 Already examined elsewhere=7

Table A3.3: Codes for recording treatment

Code	Treatment given
01	Given tablets: swallowed all
02	Given tablets: swallowed some but not all
03	Given suspension: swallowed all
04	Given suspension: swallowed some but not all
05	Offered tablets: refused; given suspension, swallowed all
06	Offered tablets: refused; given suspension, swallowed some but not all
07	Offered tablets: refused; offered suspension; refused; given tetracycl. ointment
08	Pregnant → given tetracycline ointment
09	Pregnant → offered tetracycline ointment: refused
10	Less than twelve months old → given tetracycline ointment
11	Less than twelve months old → given tetracycline ointment: refused
12	Seriously ill → unable to take medicine
55	Not pregnant, not less than twelve months old, not ill, but did not present or presented and refused all treatment

## APPENDIX 4: AZITHROMYCIN DOSAGE TABLES

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Table A4.1: Dose of azithromycin tablets, by weight

Weight in kilograms	Number of 250mg tablets
10.0 – 16.6	1
16.7 – 26.4	2
26.5 – 39.4	3
39.5 and above	4

Table A4.2: Dose of azithromycin suspension (for children who weigh less than 10kg, and heavier children who can not take tablets), by weight

Weight in kilograms	Millilitres of suspension (40mg/mL)
2.0 – 2.4	1.0
2.5 – 3.4	1.5
3.5 – 4.4	2.0
4.5 – 5.4	2.5
5.5 – 6.4	3.0
6.5 – 7.4	3.5
7.5 – 8.4	4.0
8.5 – 9.4	4.5
9.5 – 10.4	5.0
10.5 – 11.4	5.5
11.5 – 12.4	6.0
12.5 – 14.9	7.5
15.0 – 24.9	10.0
25.0 – 34.9	15.0
35.0 – 44.9	20.0
45.0 and above	25.0

## APPENDIX 5: SATSCAN OUTPUT

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SaTScan V.3.0.4

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Program run on: Sun Mar 23 08:51:08 2003

Purely Spatial analysis  
scanning for clusters with  
high rates using the Poisson model.

---

### SUMMARY OF DATA (Baseline)

Study period .....: 2000/7/11 - 2000/7/22  
Number of census areas: 205  
Total population .....: 956  
Total cases .....: 91

---

### MOST LIKELY CLUSTER

1.Census areas included.: 5808, 5809, 5810, 5811, 5801,  
5812,  
5805, 5804, 5608, 5806  
Coordinates / radius..: (9.66308e+06,344813) / 179.76  
Population.....: 51  
Number of cases.....: 17 (4.85 expected)  
Overall relative risk.: 3.502  
Log likelihood ratio..: 10.059977  
Monte Carlo rank.....: 6/1000  
P-value.....: 0.006

### SECONDARY CLUSTERS

2.Census areas included.: 4809, 4808, 4810, 4812, 4807,  
4709,  
4811, 4806, 4710  
Coordinates / radius..: (9.66362e+06,346540) / 185.17  
Population.....: 37  
Number of cases.....: 12 (3.52 expected)  
Overall relative risk.: 3.407  
Log likelihood ratio..: 6.657397  
Monte Carlo rank.....: 102/1000  
P-value.....: 0.102

3.Census areas included.: 5512, 5511  
Coordinates / radius..: (9.66308e+06,345463) / 44.08

```

Population.....: 13
Number of cases.....: 6                (1.24 expected)
Overall relative risk.: 4.849
Log likelihood ratio..: 4.838338
Monte Carlo rank.....: 434/1000
P-value.....: 0.434

4.Census areas included.: 4904, 4903
Coordinates / radius..: (9.66276e+06,346181) / 52.48
Population.....: 14
Number of cases.....: 5                (1.33 expected)
Overall relative risk.: 3.752
Log likelihood ratio..: 3.020072
Monte Carlo rank.....: 959/1000
P-value.....: 0.959

5.Census areas included.: 5011
Coordinates / radius..: (9.66301e+06,346207) / 0.00
Population.....: 6
Number of cases.....: 3                (0.57 expected)
Overall relative risk.: 5.253
Log likelihood ratio..: 2.580298
Monte Carlo rank.....: 988/1000
P-value.....: 0.988

6.Census areas included.: 5509
Coordinates / radius..: (9.66293e+06,345564) / 0.00
Population.....: 3
Number of cases.....: 2                (0.29 expected)
Overall relative risk.: 7.004
Log likelihood ratio..: 2.194735
Monte Carlo rank.....: 999/1000
P-value.....: 0.999

```

```

The log likelihood ratio value required for an observed
cluster to be significant at level
... 0.01: 9.112805
... 0.05: 7.456156

```

---

Warning: According to the input data, the following tracts have a population totalling zero for the specified year(s).

```

Tract 4613, 2000
Tract 4923, 2000
Tract 4924, 2000
Tract 5007, 2000
Tract 5018, 2000
Tract 5019, 2000
Tract 5020, 2000
Tract 5115, 2000

```

Tract 5116, 2000  
Tract 5202, 2000  
Tract 5311, 2000  
Tract 5312, 2000  
Tract 5313, 2000  
Tract 5314, 2000  
Tract 5315, 2000  
Tract 5513, 2000  
Tract 5514, 2000  
Tract 5702, 2000  
Tract 5715, 2000  
Tract 5812, 2000  
Tract 6004, 2000

---

#### PARAMETER SETTINGS

##### Input Files

-----

Case File : D:\Sol\Send\case00.txt  
Population File : D:\Sol\Send\population00\_seen.txt  
Coordinates File : D:\Sol\Send\coordinates.txt

Precision of Times : None  
Coordinates : Cartesian

##### Analysis

-----

Type of Analysis : Purely Spatial  
Probability Model : Poisson  
Scan for Areas with : High Rates

Number of Replications : 999

##### Scanning Window

-----

Maximum Spatial Cluster Size : 50.00 %

Criteria for Reporting Secondary Clusters : No  
Geographical Overlap

---

Program completed : Sun Mar 23 08:51:18 2003  
Total Running Time : 10 seconds



---

SaTScan V.3.0.4

---

Program run on: Sat Mar 22 18:42:51 2003

Purely Spatial analysis  
scanning for clusters with  
high rates using the Poisson model.

---

SUMMARY OF DATA (Two month follow-up)

Study period .....: 2000/9/19 - 2000/9/26  
Number of census areas: 205  
Total population .....: 905  
Total cases .....: 19

---

MOST LIKELY CLUSTER

1.Census areas included.: 4902, 4903  
Coordinates / radius..: (9.66281e+06,346214) / 33.83  
Population.....: 12  
Number of cases.....: 4 (0.25 expected)  
Overall relative risk.: 15.877  
Log likelihood ratio..: 7.713928  
Monte Carlo rank.....: 38/1000  
P-value.....: 0.038

SECONDARY CLUSTERS

2.Census areas included.: 5811, 5810, 5809, 5808  
Coordinates / radius..: (9.66318e+06,344821) / 99.81  
Population.....: 17  
Number of cases.....: 4 (0.36 expected)  
Overall relative risk.: 11.207  
Log likelihood ratio..: 6.404924  
Monte Carlo rank.....: 103/1000  
P-value.....: 0.103

3.Census areas included.: 4709  
Coordinates / radius..: (9.66359e+06,346407) / 0.00  
Population.....: 4  
Number of cases.....: 2 (0.08 expected)  
Overall relative risk.: 23.816  
Log likelihood ratio..: 4.525166  
Monte Carlo rank.....: 460/1000  
P-value.....: 0.460

4.Census areas included.: 5109

```

Coordinates / radius...: (9.66332e+06,345745) / 0.00
Population.....: 6
Number of cases.....: 2          (0.13 expected)
Overall relative risk.: 15.877
Log likelihood ratio...: 3.752014
Monte Carlo rank.....: 734/1000
P-value.....: 0.734

5.Census areas included.: 5511
Coordinates / radius...: (9.66305e+06,345489) / 0.00
Population.....: 9
Number of cases.....: 2          (0.19 expected)
Overall relative risk.: 10.585
Log likelihood ratio...: 2.997908
Monte Carlo rank.....: 919/1000
P-value.....: 0.919

6.Census areas included.: 5015
Coordinates / radius...: (9.66342e+06,346209) / 0.00
Population.....: 3
Number of cases.....: 1          (0.06 expected)
Overall relative risk.: 15.877
Log likelihood ratio...: 1.851441
Monte Carlo rank.....: 999/1000
P-value.....: 0.999

The log likelihood ratio value required for an observed
cluster to be significant at level
... 0.01: 9.258291
... 0.05: 7.234497

```

---

Warning: According to the input data, the following tracts have a population totalling zero for the specified year(s).

```

Tract 4609, 2000
Tract 4610, 2000
Tract 4611, 2000
Tract 4613, 2000
Tract 4707, 2000
Tract 4913, 2000
Tract 4918, 2000
Tract 4923, 2000
Tract 4924, 2000
Tract 5007, 2000
Tract 5012, 2000
Tract 5018, 2000
Tract 5019, 2000
Tract 5020, 2000
Tract 5115, 2000
Tract 5116, 2000
Tract 5202, 2000

```

Tract 5312, 2000  
Tract 5313, 2000  
Tract 5314, 2000  
Tract 5315, 2000  
Tract 5413, 2000  
Tract 5505, 2000  
Tract 5513, 2000  
Tract 5514, 2000  
Tract 5702, 2000  
Tract 5715, 2000  
Tract 5812, 2000  
Tract 6004, 2000

---

#### PARAMETER SETTINGS

##### Input Files

-----

Case File : D:\Sol\Send\case02.txt  
Population File : D:\Sol\Send\population02\_seen.txt  
Coordinates File : D:\Sol\Send\coordinates.txt

Precision of Times : None  
Coordinates : Cartesian

##### Analysis

-----

Type of Analysis : Purely Spatial  
Probability Model : Poisson  
Scan for Areas with : High Rates

Number of Replications : 999

##### Scanning Window

-----

Maximum Spatial Cluster Size : 50.00 %

Criteria for Reporting Secondary Clusters : No  
Geographical Overlap

---

Program completed : Sat Mar 22 18:43:02 2003  
Total Running Time : 11 seconds

---

SaTScan V.3.0.4

---

Program run on: Sun Mar 23 09:06:42 2003

Purely Spatial analysis  
scanning for clusters with  
high rates using the Poisson model.

---

SUMMARY OF DATA (Six month follow-up)

Study period .....: 2001/1/17 - 2001/1/24  
Number of census areas: 205  
Total population .....: 879  
Total cases .....: 13

---

MOST LIKELY CLUSTER

1.Census areas included.: 4603  
Coordinates / radius..: (9.66301e+06,346498) / 0.00  
Population.....: 4  
Number of cases.....: 2 (0.06 expected)  
Overall relative risk.: 33.808  
Log likelihood ratio..: 5.253953  
Monte Carlo rank.....: 271/1000  
P-value.....: 0.271

SECONDARY CLUSTERS

2.Census areas included.: 5511  
Coordinates / radius..: (9.66305e+06,345489) / 0.00  
Population.....: 9  
Number of cases.....: 2 (0.13 expected)  
Overall relative risk.: 15.026  
Log likelihood ratio..: 3.695130  
Monte Carlo rank.....: 702/1000  
P-value.....: 0.702

3.Census areas included.: 5018, 5019, 5006  
Coordinates / radius..: (9.66322e+06,346272) / 36.93  
Population.....: 9  
Number of cases.....: 2 (0.13 expected)  
Overall relative risk.: 15.026  
Log likelihood ratio..: 3.695130  
Monte Carlo rank.....: 716/1000  
P-value.....: 0.716

4.Census areas included.: 5710

```
Coordinates / radius...: (9.66326e+06,345204) / 0.00
Population.....: 2
Number of cases.....: 1          (0.03 expected)
Overall relative risk.: 33.808
Log likelihood ratio...: 2.587511
Monte Carlo rank.....: 985/1000
P-value.....: 0.985
```

The log likelihood ratio value required for an observed cluster to be significant at level  
... 0.01: 9.320457  
... 0.05: 7.276175

---

Warning: According to the input data, the following tracts have a population totalling zero for the specified year(s).

```
Tract 4613, 2001
Tract 4707, 2001
Tract 4920, 2001
Tract 4923, 2001
Tract 4924, 2001
Tract 5007, 2001
Tract 5013, 2001
Tract 5019, 2001
Tract 5020, 2001
Tract 5115, 2001
Tract 5116, 2001
Tract 5202, 2001
Tract 5314, 2001
Tract 5315, 2001
Tract 5404, 2001
Tract 5413, 2001
Tract 5514, 2001
Tract 5809, 2001
Tract 5903, 2001
Tract 5904, 2001
Tract 6004, 2001
```

---

#### PARAMETER SETTINGS

##### Input Files

-----

```
Case File          : D:\Sol\Send\case06.txt
Population File    : D:\Sol\Send\population06_seen.txt
Coordinates File   : D:\Sol\Send\coordinates.txt
```

```
Precision of Times : None
Coordinates         : Cartesian
```

## Analysis

-----

Type of Analysis : Purely Spatial  
Probability Model : Poisson  
Scan for Areas with : High Rates

Number of Replications : 999

## Scanning Window

-----

Maximum Spatial Cluster Size : 50.00 %

Criteria for Reporting Secondary Clusters : No  
Geographical Overlap

---

Program completed : Sun Mar 23 09:06:52 2003  
Total Running Time : 10 seconds

---

SaTScan V.3.0.4

---

Program run on: Sun Mar 23 09:17:43 2003

Purely Spatial analysis  
scanning for clusters with  
high rates using the Poisson model.

---

SUMMARY OF DATA (Twelve month follow-up)

Study period .....: 2001/7/23 - 2001/8/2  
Number of census areas: 205  
Total population .....: 907  
Total cases .....: 8

---

MOST LIKELY CLUSTER

1.Census areas included.: 5709, 5710  
Coordinates / radius..: (9.66326e+06,345153) / 52.00  
Population.....: 10  
Number of cases.....: 3 (0.09 expected)  
Overall relative risk.: 34.013  
Log likelihood ratio..: 8.285599  
Monte Carlo rank.....: 15/1000  
P-value.....: 0.015

SECONDARY CLUSTERS

2.Census areas included.: 4603  
Coordinates / radius..: (9.66301e+06,346498) / 0.00  
Population.....: 4  
Number of cases.....: 2 (0.04 expected)  
Overall relative risk.: 56.687  
Log likelihood ratio..: 6.375534  
Monte Carlo rank.....: 108/1000  
P-value.....: 0.108

3.Census areas included.: 5809, 5810  
Coordinates / radius..: (9.66313e+06,344820) / 26.59  
Population.....: 6  
Number of cases.....: 2 (0.05 expected)  
Overall relative risk.: 37.792  
Log likelihood ratio..: 5.577908  
Monte Carlo rank.....: 230/1000  
P-value.....: 0.230

The log likelihood ratio value required for an observed

cluster to be significant at level  
... 0.01: 8.679177  
... 0.05: 7.026134

---

Warning: According to the input data, the following tracts  
have a population totalling zero for the specified year(s).

Tract 4613, 2001  
Tract 4707, 2001  
Tract 4920, 2001  
Tract 4923, 2001  
Tract 5007, 2001  
Tract 5012, 2001  
Tract 5013, 2001  
Tract 5202, 2001  
Tract 5315, 2001  
Tract 5413, 2001  
Tract 5509, 2001  
Tract 5514, 2001  
Tract 5809, 2001  
Tract 5903, 2001  
Tract 5904, 2001

---

#### PARAMETER SETTINGS

##### Input Files

-----

Case File : D:\Sol\Send\case12.txt  
Population File : D:\Sol\Send\population12\_seen.txt  
Coordinates File : D:\Sol\Send\coordinates.txt

Precision of Times : None  
Coordinates : Cartesian

##### Analysis

-----

Type of Analysis : Purely Spatial  
Probability Model : Poisson  
Scan for Areas with : High Rates

Number of Replications : 999

##### Scanning Window

-----

Maximum Spatial Cluster Size : 50.00 %

Criteria for Reporting Secondary Clusters : No  
Geographical Overlap



---

Program completed : Sun Mar 23 09:17:53 2003  
Total Running Time : 10 seconds

---

SaTScan V.3.0.4

---

Program run on: Sun Mar 23 09:47:02 2003

Purely Spatial analysis  
scanning for clusters with  
high rates using the Poisson model.

---

SUMMARY OF DATA (Eighteen month follow-up)

Study period .....: 2001/12/23 - 2002/1/3  
Number of census areas: 205  
Total population .....: 888  
Total cases .....: 5

---

MOST LIKELY CLUSTER

1.Census areas included.: 5809, 5810  
Coordinates / radius..: (9.66313e+06,344820) / 26.59  
Population.....: 6  
Number of cases.....: 2 (0.03 expected)  
Overall relative risk.: 59.200  
Log likelihood ratio..: 6.649705  
Monte Carlo rank.....: 80/1000  
P-value.....: 0.080

SECONDARY CLUSTERS

2.Census areas included.: 4603  
Coordinates / radius..: (9.66301e+06,346498) / 0.00  
Population.....: 3  
Number of cases.....: 1 (0.02 expected)  
Overall relative risk.: 59.200  
Log likelihood ratio..: 3.201884  
Monte Carlo rank.....: 876/1000  
P-value.....: 0.876

3.Census areas included.: 5411  
Coordinates / radius..: (9.66332e+06,345400) / 0.00  
Population.....: 4  
Number of cases.....: 1 (0.02 expected)  
Overall relative risk.: 44.400  
Log likelihood ratio..: 2.918724  
Monte Carlo rank.....: 965/1000  
P-value.....: 0.965

4.Census areas included.: 4810

```
Coordinates / radius...: (9.66365e+06,346613) / 0.00
Population.....: 5
Number of cases.....: 1          (0.03 expected)
Overall relative risk.: 35.520
Log likelihood ratio...: 2.700108
Monte Carlo rank.....: 993/1000
P-value.....: 0.993
```

The log likelihood ratio value required for an observed cluster to be significant at level  
... 0.01: 8.025813  
... 0.05: 6.893353

---

Warning: According to the input data, the following tracts have a population totalling zero for the specified year(s).

```
Tract 4707, 2002
Tract 4913, 2002
Tract 4918, 2002
Tract 4920, 2002
Tract 5012, 2002
Tract 5013, 2002
Tract 5105, 2002
Tract 5115, 2002
Tract 5202, 2002
Tract 5303, 2002
Tract 5315, 2002
Tract 5413, 2002
Tract 5509, 2002
Tract 5809, 2002
Tract 5903, 2002
```

---

#### PARAMETER SETTINGS

##### Input Files

-----

```
Case File       : D:\Sol\Send\case18.txt
Population File  : D:\Sol\Send\population18_seen.txt
Coordinates File : D:\Sol\Send\coordinates.txt
```

```
Precision of Times : None
Coordinates         : Cartesian
```

##### Analysis

-----

```
Type of Analysis   : Purely Spatial
Probability Model    : Poisson
Scan for Areas with : High Rates
```

Number of Replications : 999

Scanning Window

-----

Maximum Spatial Cluster Size : 50.00 %

Criteria for Reporting Secondary Clusters : No  
Geographical Overlap

---

Program completed : Sun Mar 23 09:47:12 2003

Total Running Time : 10 seconds

## APPENDIX 6: TREATMENT DATA FOR KAHE VILLAGE

Table A6.1: Treatment by age, males, whole village

Age	Treatment code (see Table A3.3, p.211)												Total <sup>xxxiii</sup>
	01	02	03	04	05	06	07	08	09	10	11	12	
0-	0	0	21	0	0	0	0	0	0	76	0	0	97
1-2	42	2	137	6	5	0	0	0	0	1	0	0	193
3-4	177	2	26	1	0	0	0	0	0	0	0	0	206
5-6	185	0	13	1	1	0	0	0	0	0	0	0	200
7-8	165	0	2	0	0	0	0	0	0	0	0	0	167
9-10	190	0	3	0	0	0	0	0	0	0	0	0	193
11-15	372	0	0	0	0	0	0	0	0	0	0	0	372
16-30	149	0	0	0	0	0	0	0	0	0	0	0	149
31-60	405	0	0	0	0	0	0	0	0	0	0	0	405
60+	202	0	0	0	0	0	0	0	0	0	0	0	202
All	1887	4	202	8	6	0	0	0	0	77	0	0	<b>2184</b>

<sup>xxxiii</sup> Males who were not less than twelve months old, and not ill, but who refused all treatment or did not present do not appear in this table.

Table A6.2: Treatment by age, females, whole village

Age	Treatment code (see Table A3.3, p.211)												Total <sup>xxxiv</sup>
	01	02	03	04	05	06	07	08	09	10	11	12	
0-	1	0	32	0	0	0	0	0	0	66	0	0	99
1-2	39	0	138	13	2	1	0	0	0	0	0	0	193
3-4	186	2	23	2	0	0	0	0	0	0	0	0	213
5-6	191	0	11	0	0	0	0	0	0	0	0	0	202
7-8	177	0	2	0	0	0	0	0	0	0	0	0	179
9-10	191	0	2	0	0	0	0	0	0	0	0	0	193
11-15	392	0	1	0	0	0	0	0	0	0	0	0	393
16-30	399	0	0	0	0	0	0	36	0	0	0	0	435
31-60	658	0	1	0	0	0	0	45	0	0	0	0	704
60+	221	0	0	0	0	0	0	0	0	0	0	0	221
All	2455	2	210	15	2	1	0	81	0	66	0	0	<b>2832</b>

<sup>xxxiv</sup> Females who were not pregnant, not less than twelve months old, and not ill, but who refused all treatment or did not present do not appear in this table.

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